DEPARTAMENTO DE FARMACOLOGÍA
FACULTAD DE FARMACIA
UNIVERSIDAD DE SEVILLA

ESTUDIO QUÍMICO BIODIRIGIDO Y CARACTERIZACIÓN FARMACOLÓGICA DEL ACEITE DE OLIVA VIRGEN EXTRA EN EL LUPUS ERITEMATOSO SISTÉMICO EXPERIMENTAL

Tesis doctoral presentada por

MARINA APARICIO SOTO

Para optar al Grado de Doctor en Farmacia con Mención Internacional

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DEPARTAMENTO DE FARMACOLOGÍA
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Las Dras. Catalina Alarcón de la Lastra Romero, catedrática de Universidad, y Marina Sánchez Hidalgo, profesora contratada doctora, adscritas al departamento de Farmacología de la Facultad de Farmacia de la Universidad de Sevilla,

INFORMAN

Que la Tesis doctoral titulada "**ESTUDIO QUÍMICO BIODIRIGIDO Y CARACTERIZACIÓN FARMACOLÓGICA DEL ACEITE DE OLIVA VIRGEN EXTRA EN EL LUPUS ERITEMATOSO SISTÉMICO EXPERIMENTAL**" presentada por la Lda. Marina Aparicio Soto para optar al grado de Doctora en Farmacia con mención internacional ha sido llevaba a cabo bajo su dirección.

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Dra. Catalina Alarcón de la Lastra

Dra. Marina Sánchez Hidalgo

A mis padres

Für Tarek

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A. BECAS PREDOCTORALES

Ayudas a la realización de proyectos de investigación científica
Entidad financiadora: Instituto Danone
Duración: Enero 2012- Abril 2013

Beca de formación de profesorado universitario (F.P.U)
Entidad financiadora: Ministerio de Educación y Ciencia (M.E.C)
Duración: Mayo 2013- Abril 2017

B. ESTANCIAS EN EL EXTRANJERO

Ayudas a la movilidad del personal docente investigador (P.D.I). Tipo A
Klinik für Dermatologie, Allergologie und Venerologie. Charite-Universitätsmedizin Berlin, Alemania
Entidad financiadora: Universidad de Sevilla
Duración: Septiembre-Diciembre 2014

Ayudas a estancias cortas en el extranjero. Programa de Formación de Profesorado Universitario (F.P.U)
Klinik für Dermatologie, Allergologie und Venerologie. Charite-Universitätsmedizin Berlin, Alemania
Entidad financiadora: Ministerio de Educación y Ciencia (M.E.C)
Duración: Septiembre 2015-Febrero 2016

C. PROYECTOS DE INVESTIGACIÓN

Plan Nacional del 2011: Estudio químico biodirigido y caracterización farmacológica del aceite de oliva virgen extra en el lupus eritematoso sistémico experimental.
Investigador principal: Catalina Alarcón de la Lastra
Referencia: AGL2011-26949
Entidad financiadora: Ministerio de Ciencia e Innovación
Duración: 2012-2015

ABREVIATURAS

A

AD	Atopic dermatitis
7-ADD	7-aminoactinomycin D
AG	Aceite de girasol
AIN	American Institute of Nutrition
Akt	Protein kinase B
ALS	Amyotrophic lateral sclerosis
AMPK	Adenosine 5'-monophosphate-activated protein kinase
ANA	Antinuclear antibodies
ANP	Atrial natriuretic peptide
Anti-dsDNA	Anti-double-stranded DNA
AO	Aceite de oliva
AOVE	Aceite de olive virgen extra
AP-1	Activator protein 1
AR	Artritis reumatoide

B

BAFF	B-cell activating factor
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin

C

CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CIA	Collagen-induced arthritis / Artritis inducida por colágeno tipo II
COMP	Cartilage olimeric matrix protein

Abreviaturas

Con-A	Concanavalin-A
COX-2	Cyclooxygenase-2 / Ciclooxigenasa-2
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4C-X-C
CXC	CXC motif chemokine
CXCR	Motif chemokine receptor

D

DC	Dendritic cells
DHA	Docosahexaenoic acid
DHPG	3,4-dihidroxyphenylglycol
MEM	Minimal essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2- diphenyl-1-picrylhydrazyl
DSS	Dextrane sulphate sodium
DTT	Dithiothreitol

E

EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EGF	Endothelial grow factor
ELISA	Enzyme-linked immunoassay
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinases / Cinasa regulada

	por señal extracelular
ER	Oestrogen receptors
EU	European Union
EVOO	Extra virgin olive oil

F

FBS	Foetal bovine serum
FI	Fracción insaponificable
FOXP3	Forkhead box P3
FP	Fracción polifenólica

G

GC	Gas chromatography
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GST	Glutathione-S-transferase
Grp78	78 kDa glucose-regulated protein

H

H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
H&E	Hematoxylin and eosin
HO-1	Heme oxygenase-1

Abreviaturas

HRP	Horseradish peroxidase
HTy	Hydroxytyrosol/ Hidroxitirosol
HTy-Ac	Hydroxytyrosyl acetate/ Acetato de hidroxitirosol
	I
IBD	Inflammatory bowel disease
I3C	Indole-3-carbinol
IC	Aberrant immune complexes/ Inmunocomplejos aberrantes
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon / Interferón
Ig	Immunoglobulin
IGF1R	Insulin-like growth factor receptor 1
IκBα	Inhibitor of NF-κB
IL	Interleukin / Interleucina
iNOS	Inducible nitric oxide synthase / Óxido nitrico sintasa Inducible
i.p.	Intraperitoneally / Intraperitoneal
IRF-1	Interferon regulatory factor-1
IU	International units
i.v.	Intravenous / Intravenoso
	J
JAK/STAT	Janus kinase-signal transducer and activator of Transcription/ Janus quinasas-transductor de señal y activador de la transcripción
JNK	c-Jun N H_2-terminal kinase / C-Jun NH_2-terminal cinasa

L

LDL	Low-density lipoproteins
LES	Lupus eritematoso sistémico
LFA	Lymphocyte function-associated antigen 1
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LPO	Lipid peroxides

M

MAPK	Mitogen-activated protein kinases / Proteínas cinasas activadas por mitógenos
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Metalloproteinases / Metaloproteinasas
MOG	Myelin oligodendrocyte glycoprotein
MPC-1	Monocyte chemoattractant protein-1
mPGES-1	Microsomal prostaglandin E synthase-1
MPO	Myeloperoxidase
mRNA	Messenger RNA
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium
MUFA	Monounsaturated fatty acid / Ácidos grasos monoinsaturados

Abreviaturas

N

NADH	Nicotinamide adenine dinucleotide
NF-κB	Nuclear transcription factor-kappa B / Factor nuclear kappa B
NK	Natural killer cell
NLRP3	NLR family pyrin domain-containing 3 inflammasome/ Inflamasoma NLR3
NO	Nitric oxide/ Óxido nítrico
Nrf2	Nuclear factor E2-related factor 2/ Factor nuclear eritroide 2

O

OA	Oleanolic acid
OD	Optical density
OL	Oleuropein
OL-aglycon	Oleuropein aglycone

P

pAKT	Phospho AKT
PAS	Periodic acid Schiff
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PE	Phenolic fraction
PG	Prostaglandin/ Prostaglandina
PHA	Phytohaemagglutinin/ Fitohemaglutinina

PI3	Phosphatidylinositol-3
PI3K-Akt-mTOR	phosphoinositide 3-kinase/Akt/mammalian target of rapamycin
PKC	Protein kinase C
PMSF	Phenylmethyl sulfonyl fluoride
p.o	Oral administration (*per os*)
PPAR	Peroxisome proliferator-activated receptor/ Receptores activados por proliferadores de peroxisomas
ppm	Parts per million
PUFA	Polyunsaturated fatty acids
	R
RA	Rheumatoid arthritis
RANKL	Receptor activator of NF-κB ligand
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription polymerase chain reaction/ Reacción en cadena de la polimerasa con transcriptasa inversa
	S
SD	Standard diet
SDG	Secoisolariciresinol diglucoside
SDS	Sodium dodecyl sulfate
S.E.M.	Standard error
SOD	Superoxide dismutase

Abreviaturas

SRB	Sulforhodamine B
ssDNA	Anti-single-stranded DNA
STAT	Signal transducer and activator of transcription
	T
TG	Triglycerides
TGF	Transforming growth factor
Th	Helper T cells / Células T cooperadoras
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TLR	Toll like receptor
TLR3-I	Toll ike receptor 3 ligand/ Ligando de receptor de tipo toll 3
TSLP	Thymic stromal lymphopoietin/ Linfopoyetina estromal tímica
TNF	Tumour necrosis factor / Factor de necrosis tumoral
Tregs	Regulatory T cells / Células T reguladoras
	U
UF	Unsaponifiable fraction
UGT	Uridine diphosphate glucuronosyltransferase
UVB	Ultraviolet B
	V
VEGF	Vascular endothelial growth factor
VLDL	Low-density lipoprotein cholesterol
VOO	Virgin olive oil

W

WST-8	(2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt)

INDICE

INTRODUCTION

AN UPDATE ON DIET AND NUTRITIONAL FACTORS IN SYSTEMIC LUPUS ERYTHEMATOSUS MANAGEMENT

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UNA ACTUALIZACIÓN DE LOS FACTORES DIETÉTICOS Y NUTRICIONALES EN EL MANEJO DEL LUPUS ERITEMATOSO SISTÉMICO

Marina Aparicio-Soto, Marina Sánchez-Hidalgo, Catalina Alarcón-de-la-Lastra

Departamento de Farmacología, Facultad de Farmacia, Universidad de Sevilla, España.

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M. Aparicio-Soto, M. Sánchez-Hidalgo, C. Alarcón-de-la-Lastra. An update on diet and nutritional factors in systemic lupus erythematosus management. Nut Research Reviews (2016).

RESUMEN

El lupus eritematoso sistémico (LES) es una enfermedad autoinmune inflamatoria crónica caracterizada por afectar a múltiples órganos del organismo y presentar un amplio número de complicaciones. En la actualidad, el manejo del LES continua siendo complicado debido a las notables diferencias que existen entre pacientes con LES, así como a la falta de terapias efectivas y seguras. Existen evidencias que ponen de manifiesto que diversos factores dietéticos contribuyen a la geoepidemiología de diversas enfermedades autoinmunes, incluyendo el LES. Por lo tanto, la terapia nutricional puede constituir un interesante abordaje del LES, debido a sus potenciales efectos profilácticos sin los efectos adversos asociados a la farmacoterapia clásica, contribuyendo por tanto a reducir morbilidades y mejorar la calidad de vida de los pacientes con LES. Sin embargo, la cuestión a resolver es si distintos nutrientes pueden mejorar o exacerbar el LES y como dichos nutrientes podrían modular el sistema inmune y el proceso inflamatorio. En el presente trabajo se recogen evidencias preclínicas y clínicas, aportando una actualización de los aspectos positivos y negativos de micro y macro nutrientes de la dieta y de otros factores nutricionales, incluyendo el consumo de polifenoles, en el LES, prestando especial atención a los mecanismos de acción involucrados.

AN UPDATE ON DIET AND NUTRITIONAL FACTORS IN SYSTEMIC LUPUS ERYTHEMATOSUS MANAGEMENT

Marina Aparicio-Soto, Marina Sánchez-Hidalgo, Catalina Alarcón-de-la-Lastra

Department of Pharmacology, Faculty of Pharmacy, University of Seville, Spain

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ABSTRACT

Systemic lupus erythematosus (SLE) is a chronic inflammatory and autoimmune disease characterised by multiple organ involvement and a large number of complications. SLE management remains complicated owing to the biological heterogeneity between patients and the lack of safe and specific targeted therapies. There is evidence that dietary factors can contribute to the geoepidemiology of autoimmune diseases such as SLE. Thus, diet therapy could be a promising approach in SLE owing to both its potential prophylactic effects, without the side effects of classical pharmacology, and its contribution to reducing comorbidities and improving quality of life in patients with SLE. However, the question arises as to whether nutrients could ameliorate or exacerbate SLE and how they could modulate inflammation and immune function at a molecular level. The present review summarises preclinical and clinical experiences to provide the reader with an update of the positive and negative aspects of macro and micronutrients and other nutritional factors, including dietary phenols, on SLE, focusing on the mechanisms of action involved.

Keywords: diet, immunomodulation, lupus, nutrient, SLE.

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EXTRA VIRGIN OLIVE OIL: A KEY FUNCTIONAL FOOD FOR PREVENTION OF IMMUNE-INFLAMMATORY DISEASES

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EL ACEITE DE OLIVA VIRGEN EXTRA: UN ALIMENTO FUNCIONAL CLAVE EN LA PREVENCIÓN DE ENFERMEDADES INMUNOINFLAMATORIAS

Marina Aparicio-Soto, Marina Sánchez-Hidalgo, Mª Ángeles Rosillo, Mº Luisa Castejón, Catalina Alarcón-de-la-Lastra

Departamento de Farmacología, Facultad de Farmacia, Universidad de Sevilla, España.

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RESUMEN

En la actualidad se considera que una dieta desequilibrada constituye uno de los principales factores que contribuyen a la aparición de enfermedades inflamatorias y autoinmunes tanto en países desarrollados como en países en vías de desarrollo. Por el contrario, la dieta mediterránea ha sido especialmente asociada con una disminución en la incidencia de ciertas patologías relacionadas con la inflamación crónica y el sistema inmune. En este sentido, el aceite de oliva, la principal fuente de lípidos de la dieta mediterránea, presenta excepcionales cualidades nutricionales, así como una especial composición particularmente destacable en el caso del aceite de oliva virgen extra (AOVE), obtenido a partir de la aceituna únicamente a través de procedimientos mecánicos o físicos bajo una serie de condiciones que no alteran la composición natural del aceite. El AOVE ha sido ampliamente descrito como un alimento funcional de primera línea con múltiples propiedades beneficiosas, que podrían ser de interés en el tratamiento de diversas enfermedades inmunoinflamatorias. En el presente trabajo, se recogen los resultados clave que aportan evidencias sobre los efectos beneficiosos del AOVE y sus componentes minoritarios, prestando especial interés a los mecanismos por los cuales pueden ejercer dichos efectos beneficiosos en el manejo de diversas enfermedades inmunoinflamatoria como la artritis reumatoide, el lupus eritematoso sistémico, la enfermedad inflamatoria intestinal y la esclerosis.

EXTRA VIRGIN OLIVE OIL: A KEY FUNCTIONAL FOOD FOR PREVENTION OF IMMUNE-INFLAMMATORY DISEASES

Marina Aparicio-Soto, Marina Sánchez-Hidalgo, Mª Ángeles Rosillo, Mº Luisa Castejón, Catalina Alarcón-de-la-Lastra

Department of Pharmacology, Faculty of Pharmacy, University of Seville, Spain

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ABSTRACT

Nowadays, it is clear that unhealthy diet is one of the prime factors that contribute to the rise of inflammatory diseases and autoimmunity in both developed and developing countries. The Mediterranean diet has been associated with a reduced incidence of certain pathologies related to chronic inflammation and immune system. Olive oil, the principal source of dietary lipids of the Mediterranean diet, possesses a high nutritional quality and a particular composition, which is especially relevant in the case of Extra Virgin Olive Oil (EVOO). EVOO is obtained from olives solely by mechanical or other physical preparation under conditions that do not alter natural composition. EVOO is described as a key bioactive food with multiple beneficial properties and it may be effective in the management of some immune-inflammatory diseases. In this review, we summarise the key works, which provided evidence of the beneficial effects of EVOO and its minor components focusing on their mechanisms on immune-inflammatory diseases like rheumatoid arthritis, systemic lupus erythematous, inflammatory bowel disease and sclerosis.

Keywords: EVOO, olive oil, inflammation, autoimmune disease, functional food

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JUSTIFICACIÓN Y OBJETIVOS

El lupus eritematoso sistémico (LES) es una enfermedad autoinmune inflamatoria crónica que puede afectar a múltiples órganos, provocando un deterioro progresivo que afecta a la calidad de vida de los pacientes. Esta enfermedad se caracteriza por su complejidad clínica y patogénica, su difícil diagnóstico y el importante número de complicaciones que pueden surgir en el seguimiento de los individuos, lo que obliga a una vigilancia constante y sobre todo a una concepción global del paciente y su enfermedad (Noble et al., 2016). El curso de la enfermedad es impredecible, con periodos de exacerbaciones y remisiones, pudiendo afectar a diferentes sistemas del organismo. Las manifestaciones clínicas más habituales son fatiga, pérdida de apetito y peso, afectación cutánea, dolores articulares, serositis (pleuritis y/o pericarditis), afectación renal y del sistema nervioso, junto con manifestaciones hematológicas (citopenias), y un mayor riesgo de aterosclerosis y eventos vasculares (Lisnevskaia et al., 2014; Podolska et al., 2015).

El LES es considerado como una enfermedad rara según la denominación de ésta por la Comisión Europea "*Regulation on Orphan Medicinal Products*". Demográficamente, la prevalencia se encuentra entre 20 y 150 casos por cada 100000 habitantes de la población mundial, con una amplia variabilidad geográfica y étnica. Esta enfermedad puede afectar a ambos sexos, pero aproximadamente en un 90% de los casos aparece en mujeres en edad fértil. Además, aunque los síntomas pueden presentarse a cualquier edad, la mayoría de los casos se manifiestan entre el final de la segunda década de vida del paciente y el principio de la tercera (Vina et al., 2014).

En la actualidad aún se desconoce la etiología de LES, aunque factores genéticos, medioambientales (estrés extremo, exposición a rayos ultravioleta, ciertas infecciones o tabaco), la administración de ciertos medicamentos (algunos antidepresivos y antibióticos) y hormonales (estrógenos) parecen estar involucrados en la aparición y progresión de la enfermedad (Cai et al., 2014; Mak and Tay, 2014; Mirabelli et al., 2015). Hasta el momento, se han identificado múltiples genes que contribuyen a la predisposición y susceptibilidad del LES incluyendo *IRF5*, *STAT4*, osteopontina, *IRAK1*, *TREX1* y *TLR8*, así como genes relacionados con la producción de interferón (IFN) o implicados en vías de señalización mediadas por células T (*PTPN22, TNFSF4, PDCD1*) y células B (*BANK1, BLK, LYN*). Asimismo, estudios recientes han puesto de manifiesto que varias regiones localizadas en el complejo mayor de histocompatibilidad contribuyen a incrementar el riesgo de padecer LES (Moser et al., 2009; Ruiz-Larrañaga et al., 2016; Xiong et al., 2014).

Desde el punto de vista patológico, el LES es una enfermedad inflamatoria crónica que se caracteriza por la pérdida de autotolerancia de las células T y B con la consiguiente hiperactividad linfocítica, la producción de autoanticuerpos (antinucleares,

antiDNA de doble cadena, anti-Ro, antihistona anti-Smith y antiribonucleoproteina, entre otros), la formación de inmunocomplejos aberrantes (ICs), y la generación de una respuesta inflamatoria que puede afectar a múltiples órganos (Manson and Isenberg, 2003).

Los autoanticuerpos, ICs, y células T penetran en los tejidos diana donde, con la colaboración de citoquinas proinflamatorias contribuyen al daño tisular. Estos procesos incluyen el desbalance de la producción de citoquinas Th1, Th2 y Th17. Concretamente, en pacientes con LES activo suelen detectarse altos niveles de interferón (IFN)-γ, factor de necrosis tumoral (TNF)-α, interleucinas (IL)-4, IL-6, IL-10, IL-12, IL-17 y IL-18 por el contrario, se observan concentraciones más bajas de IL-2 que en individuos sanos (Chun et al., 2007; Dolff et al., 2011). Igualmente, las metaloproteinasas de matriz (MMP) son interesantes biomarcadores de esta enfermedad y se encuentran sobre-expresadas en el suero de estos pacientes (Gheita et al., 2015).

Las vías de señalización molecular involucradas en el LES incluyen la vía del factor nuclear potenciador de las cadenas ligeras kappa de células B activadas (NF-κB), la vía de proteínas janus quinasas/transductor de señal y activador de la transcripción (JAK/STAT), la vía de las proteínas quinasas activadas por mitógenos (MAPK), y más recientemente la familia del inflamasoma (NLRP3), y la vía del factor nuclear eritroide 2 (Nrf2) (Bloch et al., 2014; Jiang et al., 2014; Kawasaki et al., 2011).

En la actualidad no se dispone de un tratamiento efectivo y eficaz para el LES, por lo que el objetivo principal de la terapia es el control de los síntomas de la enfermedad. En pacientes con baja actividad, los síntomas pueden ser controlados mediante antiinflamatorios no esteroideos y bajas dosis de corticoides, pero en casos más severos se requiere de un tratamiento mas avanzado. El abordaje terapéutico básico consiste en una combinación de fármacos antipalúdicos (principalmente hidroxicloroquina y cloroquina), medicamentos inmunosupresores y biológicos, junto con algunas terapias coadyuvantes, siguiendo recomendaciones internacionales (Kuhn et al., 2015). Aunque el tratamiento del LES ha mejorado en las ultimas décadas y nuevos agentes se encuentran en desarrollo, en la actualidad tanto el LES como sus tratamientos contribuyen a incrementar la mortalidad y morbilidad de la enfermedad y el pronóstico permanece negativo en un considerable número de enfermos (Postal et al., 2016).

El abordaje terapéutico del LES continua siendo complicado debido a las importantes diferencias que aparecen entre pacientes así como a la falta de terapias efectivas y seguras. Es por ello que en la actualidad, uno de los principales focos de la

investigación del LES es la búsqueda de terapias menos tóxicas y más selectivas, que puedan mejorar el curso de la enfermedad sin generar efectos adversos.

La terapia nutricional, incluyendo modificaciones de la dieta y el uso de suplementos nutricionales, puede ser un prometedor enfoque en el abordaje del LES, ya que más allá de su soporte dietético presenta potenciales efectos profilácticos sin los efectos adversos asociados a la farmacoterapia clásica, contribuyendo a reducir comorbilidades y mejorar la calidad de vida de los pacientes con LES (Greco et al., 2013). En los últimos años, se ha puesto de manifiesto el potencial terapéutico de los alimentos funcionales: que se definen como aquellos alimentos que independientemente de sus propiedades nutritivas poseen un efecto beneficioso para el organismo, es decir, su ingesta diaria, dentro de una dieta equilibrada, contribuye a mantener o mejorar el estado de salud y bienestar.

En este sentido, recientes estudios han confirmado que el consumo habitual de aceite de oliva (AO), alimento funcional de primera magnitud dentro del contexto de la dieta mediterránea, es eficaz en la prevención y tratamiento de ciertas patologías relacionadas con el estrés oxidativo, la inflamación crónica y el sistema inmune (Alarcón de la Lastra et al., 2001; Martín-Peláez et al., 2013; Vargas et al., 2016). En particular, el consumo de AO ha demostrado efectos beneficiosos en enfermedades cardiovasculares, digestivas, envejecimiento y diversos tipos de cáncer (principalmente cáncer colorrectal, pulmón, estómago y mama), así como en enfermedades reumáticas (Buckland et al., 2012; Escrich et al., 2011; Loued et al., 2013; Servili et al., 2013). En base a estos resultados, el AO puede definirse desde un punto de vista estrictamente inmunológico como un componente de la dieta capaz de ejercer un efecto inmunomodulador (Puertollano et al., 2010).

El AO es obtenido del fruto del olivo (*Olea Europaea* L.) por un proceso que incluye principalmente operaciones mecánicas, uso de disolventes y procesos de esterificación. En el mercado podemos encontrar distintos tipos de AO en base a diferentes parámetros analíticos de calidad regulados por normativas europeas como son el grado de acidez, índice de peróxidos, absorbancia en el ultravioleta, características organolépticas y contenidos en ceras. Según el reglamento de la comunidad europea (UE nº 1348/2013; UE nº 29/2012; CEE nº 1513/2001 y CE nº 1989/2003) los AO se clasifican en aceite de oliva virgen extra (AOVE), aceite de oliva virgen, aceite de oliva virgen lampante, aceite de oliva refinado, aceite de oliva y aceites de orujo (Figura 1).

El AOVE es obtenido de la aceituna tras aplicar únicamente procedimientos mecánicos o medios físicos en condiciones sobre todo térmicas y cuya acidez libre,

expresada en gramos de ácido oleico, es como máximo un 0.8%. El AOVE está constituido por una fracción mayoritaria saponificable, que constituye entre un 90-99% del total del aceite y por una fracción minoritaria compuesta por dos subfracciones: fracción insaponificable (FI) y fracción polifenólica (FP). Los principales componentes de la fracción saponificable son ácidos grasos, de los cuales el ácido graso monoinsaturado (MUFA) ácido oleico (n-9) constituye hasta un 80% del total de la composición lipídica. Además, la fracción saponificable contiene ácidos grasos poliinsaturados, en un porcentaje del 3 al 22%, principalmente acido linoleico, y ácidos grasos saturados, en un porcentaje del 8 al 26%, principalmente acido palmítico. Por otra parte, la FI está constituida principalmente por hidrocarburos, principalmente escualeno aunque también contiene esteroles, alcoholes y dioles terpénicos y alifáticos (eritrodiol, uvaol, ácido oleanólico entre otros), vitaminas (α, β, γ y δ tocoferoles), pigmentos (clorofila y carotenos) y compuestos volátiles responsables de las propiedades organolépticas del AO (Ghanbari et al., 2012).

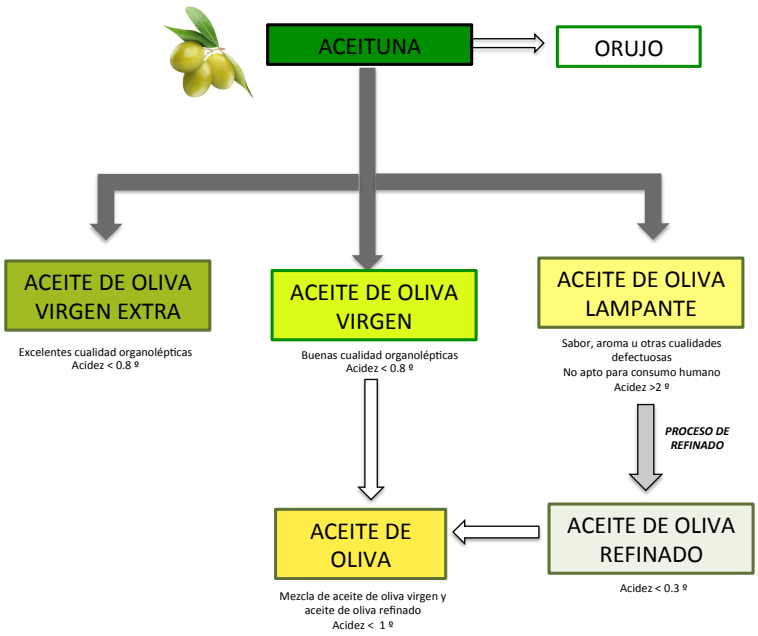

Figura 1. Tipos de aceite de oliva según reglamentos de la Comunidad Europea

La FP del AOVE es una mezcla compleja de compuestos fenólicos que contribuyen a las propiedades organolépticas del aceite. Su contenido varia considerablemente en función de la variedad del fruto y de las condiciones de cultivo y procesamiento del mismo. Al menos 36 compuestos fenólicos distintos han sido

identificados en la FP del AOVE, principalmente ácidos fenólicos y otros compuestos bioactivos como el hidroxitirosol (HTy), oleuropeína, oleaceína, oleuropeína aglicona, oleocantal, apigenina, (+)-pinoresinol y (+)-1-acetoxipinoresinol, entre otros (Pérez et al., 2014).

Hasta hace relativamente poco tiempo, las propiedades beneficiosas del AO habían sido atribuidas casi exclusivamente a su alto contenido en MUFAs, particularmente, ácido oleico. En este sentido, recientes estudios han puesto de manifiesto que los MUFAs son capaces de modular diversos parámetros inmunológicos, desempeñando un importante papel en la regulación de la respuesta inmune, por lo que podrían ser de gran interés en el tratamiento de enfermedades inmunoinflamatorias y en la regulación general del sistema inmune (Carrillo et al., 2012; Sales-Campos et al., 2013). Sin embargo, también existen evidencias contradictorias sobre el papel de los MUFA en la respuesta inflamatoria y la modulación del sistema inmune (Cury-Boaventura et al., 2008; Hatanaka et al., 2006).

Este hecho ha propiciado la búsqueda de otros posibles componentes del AO con actividad antiinflamatoria e inmunomoduladora. Existen evidencias que demuestran que los componentes minoritarios presentes en el AOVE también contribuyen a los beneficios asociados al consumo del mismo. En este sentido, AOs de baja calidad (aceites de oliva refinados) presentan una menor capacidad antioxidante y antiinflamatoria, posiblemente debido a la falta de estos componentes minoritarios (Estruch and Salas-Salvadó, 2013). Por lo tanto, los compuestos fenólicos del AOVE parecen ser responsables de algunas de sus propiedades beneficiosas, incluyendo sus actividades antiaterogénica, hipoglucemiante, antiinflamatoria, antitumoral, antiviral e inmunomodulatoria, que aparecen estar relacionadas solo parcialmente con la capacidad antioxidante intrínseca de estos compuestos (Rigacci and Stefani, 2016). Particularmente, en estudios realizados en humanos ,AOVEs ricos en compuestos fenólicos han demostrado mejores actividades antioxidante y antiinflamatoria comparados con AO de bajo contenido en compuestos fenólicos (Covas, 2007; Medina-Remón et al., 2016). Asimismo, recientes publicaciones de nuestro grupo de investigación han confirmado los efectos antiinflamatorios e inmunomoduladores de las fracciones FP y FI, así como de compuestos fenólicos aislados presentes en el AOVE en enfermedades inmunoinflamatorias en particular la enfermedad inflamatoria intestinal y la artritis reumatoide y algunos tipos de cáncer como el cáncer colorrectal así como en modelos de inflamación *in vitro* (Cardeno et al., 2014; Rosillo et al., 2015a; Rosillo et al., 2015b; Sanchez-Fidalgo et al., 2013; Sánchez-Fidalgo et al., 2010). Estos resultados sugieren que la ingesta de AOVE a través de la dieta, así como la suplementación dietética con

fracciones del AOVE o con compuestos fenólicos podría constituir una interesante estrategia para el tratamiento nutricional de patologías relacionadas con la inflamación y el sistema inmune, como es el caso del LES.

A pesar del esfuerzo investigador llevado a cabo en los últimos años, aún se desconoce el potencial antiinflamatorio e inmunomodulador del AOVE y de sus componentes bioactivos en el LES. Previamente, diversos estudios preclínicos y clínicos han puesto de manifiesto que el consumo regular de AOVE dentro de la dieta mediterránea posee diversas propiedades beneficiosas que podrían ser de interés en el manejo del LES, debido a su papel cardioprotector, antiinflamatorio, antiartrítico e inmunomodulador (Covas et al., 2015; Miggiano and Gagliardi, 2005; Perez-Jimenez et al., 2005). No obstante, hasta el momento no existían estudios experimentales concluyentes que validasen los posibles beneficios concretos del AOVE o de sus componentes bioactivos en el desarrollo y progresión de esta enfermedad autoinmune, así como de los mecanismos bioquímicos y moleculares involucrados.

En vista a estos antecedentes, el **objetivo general** de la presente tesis doctoral ha sido investigar el funcionalismo del AOVE en el LES experimental. Para ello, nos hemos centrado en una serie de objetivos específicos:

1. Valoración del potencial inmunomodulador de una dieta elaborada con AOVE en un modelo experimental *in vivo* de LES inducido por pristano (2,6,10,14-tetrametilpentadecano) en ratones BALB/c. Estudio de las rutas bioquímicas y mecanismos de señalización molecular implicados más significativos.

2. Caracterización del protagonismo de la FP del AOVE seleccionado así como los mecanismos y vías de señalización implicadas en modelos *in vitro* de:
   2a. Monocitos y macrófagos de individuos sanos
   2b. Células mononucleares de sangre periférica de pacientes con LES y controles sanos.

3. Determinación del papel modulador y antiinflamatorio de compuestos fenólicos presentes en la FP del AOVE, hidroxitirosol (HTy) y acetato de hidroxitirosol (HTy-AC):
   3a. Valoración de la actividad antiinflamatoria e inmunomoduladora de HTy, HTy-Ac y otros compuestos fenólicos del AOVE en un modelo *in vitro* de macrófagos peritoneales murinos.

3b. Valoración del potencial inmunomodulador de dietas enriquecidas con HTy o HTy-Ac en un modelo experimental *in vivo* de LES inducido por pristano (2,6,10,14-tetrametilpentadecano) en ratones BALB/c. Dilucidar las rutas bioquímicas y mecanismos de señalización molecular implicados.

3c. Determinación del posible efecto antiinflamatorio de HTy e HTy-Ac en un modelo experimental *in vitro* de queratinocitos humanos aislados de piel de individuos sanos. Estudio de la modulación génica, producción de citoquinas y vías de señalización implicadas.

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CHAPTER I

DIETARY EXTRA VIRGIN OLIVE OIL ATTENUATES KIDNEY INJURY IN PRISTANE-INDUCED SLE MODEL VIA ACTIVATION OF HO-1/NRF2 ANTIOXIDANT PATHWAY AND SUPPRESSION OF JAK/STAT, NF-κB AND MAPK ACTIVATION

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EL ACEITE DE OLIVA VIRGEN EXTRA DISMINUYE EL DAÑO RENAL EN UN MODELO MURINO DE LUPUS ERITEMATOSO SISTEMICO INDUCIDO POR PRISTANO.

Marina Aparicio-Soto[1], Marina Sánchez-Hidalgo[1], Ana Cárdeno[1], María Ángeles Rosillo[1], Susana Sánchez-Fidalgo[1], José Utrilla[2], Inés Martín-Lacave[2], Catalina Alarcón-de-la-Lastra[1]

[1]Departamento de Farmacología, Facultad de Farmacia, Universidad de Sevilla, España.
[2]Departamento de Citología e Histología, Facultad de Medicina, Universidad de Sevilla, España.

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RESUMEN

El lupus eritematoso sistémico (LES) es una enfermedad autoinmune caracterizada por una amplia y diversa afectación del organismo. Recientes estudios han puesto de manifiesto que el aceite de oliva virgen extra (AOVE) puede presentar efectos preventivos en el desarrollo de esta enfermedad inmunoinflamatoria. Sin embargo, en la actualidad se desconoce el posible efecto beneficioso del AOVE en el LES. En el presente estudio se evalúan los efectos de una dieta elaborada con AOVE en un modelo murino de LES inducido por pristano. Para ello se utilizaron ratones de 3 meses de edad, los cuales fueron inyectados con pristano o solución salina y alimentados con dietas elaboradas con aceite de girasol o AOVE. Tras 24 semanas de desarrollo de la enfermedad, los animales fueron sacrificados y bazos y riñones fueron extraídos. La expresión renal de diversas proteínas inflamatorias involucradas en el daño renal fue evaluada mediante western blot y los niveles de metaloproteinasa (MMP)-3 en suero y de citoquinas inflamatorias en riñón y esplenocitos fueron determinadas por ELISA. Nuestro estudio reveló que aquellos animales alimentados con dietas elaboradas con AOVE presentaban una significativa reducción del daño renal inducido por pristano así como menores niveles de MMP-3 en suero y prostaglandina E_2 en riñón. Además, la dieta elaborada con AOVE mejoró la expresión de diversas proteínas implicadas en el daño renal . Estos resultados demuestran el interés del AOVE como un alimento funcional de primera categoría, que puede ejercer un papel preventivo/paliativo en el manejo del SLE.

DIETARY EXTRA VIRGIN OLIVE OIL ATTENUATES KIDNEY INJURY IN PRISTANE-INDUCED SLE MODEL VIA ACTIVATION OF HO-1/NRF2 ANTIOXIDANT PATHWAY AND SUPPRESSION OF JAK/STAT, NF-KB AND MAPK ACTIVATION.

Marina Aparicio-Soto[1], Marina Sánchez-Hidalgo[1], Ana Cárdeno[1], María Ángeles Rosillo[1], Susana Sánchez-Fidalgo[1], Jose Utrilla[2], Inés Martín-Lacave[2], Catalina Alarcón-de-la-Lastra[1]

[1]Department of Pharmacology, Faculty of Pharmacy, University of Seville, Spain
[2]Department of Cytology and Histology, School of Medicine, University of Seville, Spain.

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ABSTRACT

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by a widespread organ involvement. Recent studies have suggested that extra virgin olive oil (EVOO) might possess preventive effects on this immunoinflammation-related related disease. However, its role in SLE remained unknown. In this work, we evaluated the effects of EVOO diet in a pristane-induced SLE model in mice. Three-month old mice received an injection of pristane or saline solution and were fed with different experimental diets: sunflower oil diet or EVOO diet. After 24 weeks, mice were sacrificed, spleens were collected and kidneys were removed for immune-inflammatory detections. The kidney expression of microsomal prostaglandin E synthase-1, heme oxigenase-1 (HO-1), nuclear factor E2-related factor 2 (Nrf2), mitogen-activated protein kinases (MAPK), Janus kinase/signal transducer and activator of transcription (JAK/STAT) and nuclear transcription factor-kappa B (NF-κB) pathways were studied by western blotting. In addition to macroscopic and histological analyses, serum metalloproteinase 3 (MMP-3) levels and proinflammatory cytokines production in splenocytes were evaluated by enzyme-linked immunoassay. We have demonstrated that EVOO diet significantly reduced renal damage and decreased MMP-3 serum and PGE_2 kidney levels as well as the proinflammatory cytokines production in splenocytes. Our data indicate that Nrf2 and HO-1 protein expressions were upregulated in those mice fed with EVOO and the activation of JAK/STAT; MAPK and NF-κB pathways were drastically ameliorated. These results support

the interest of EVOO as a beneficial functional food exerting a preventive/palliative role in the management of SLE.

Keywords: EVOO, immunomodulation, inflammation, SLE, polyphenols

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CHAPTER II

POTENTIAL INVOLVEMENT OF THE PHENOLIC FRACTIONOF EXTRA VIRGIN OLIVE OIL IN PRISTANE-INDUCED LUPUS IN MICE BY REGULATING PPAR-γ DEPENDENT MONOCYTE ACTIVATION

POSIBLE IMPLICACIÓN DE LA FRACIÓN POLIFENÓLICA DEL ACEITE DE OLIVA VIRGEN EXTRA EN UN MODELO MURINO DE LUPUS A TRAVÉS DE LA REGULACIÓN DE LA ACTIVACIÓN MONOCITARIA DEPENDIENTE DE PPAR-γ

Marina Aparicio-Soto[1], Sergio Montserrat de la Paz[2], Marina Sánchez-Hidalgo[1], Ana Cárdeno[1], Beatriz Bermúdez[2], Francisco J. Garcia Muriana[2], Catalina Alarcón-de-la-Lastra[1]

[1]Department of Pharmacology, Faculty of Pharmacy, University of Seville, Spain
[2]Laboratory of Cellular and Molecular Nutrition, Instituto de la Grasa, CSIC, Seville, Spain.

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RESUMEN

El lupus eritematoso sistémico (LES) es una enfermedad autoinmune caracterizada por la pérdida del equilibrio y control de la regulación celular del organismo. Monocitos y macrófagos tienen un papel fundamental en la regulación del proceso inflamatorio y la inmunidad innata. Recientes estudios han sugerido que el aceite de oliva virgen extra (AOVE) podría presentar efectos preventivos y paliativos en enfermedades inmunoinflamatorias. En el presente trabajo se evaluaron los efectos de una dieta elaborada con AOVE en macrófagos peritoneales procedentes de ratones con LES inducido por pristano. Además se evaluó el efecto de la fracción polifenólica (FP) del AOVE en un modelo *in vitro* de monocitos humanos y macrófagos derivados de monocitos. Para ello, ratones BALB/c fueron inyectados con pristano o solución salina y alimentados durante 6 meses con dietas experimentales elaboradas con AOVE o aceite de girasol. Tras el periodo experimental, los animales fueron sacrificados y los macrófagos peritoneales fueron extraídos para la determinación de mediadores inflamatorios. Para el estudio *In* vitro de los efectos de la FP se utilizaron monocitos y macrófagos derivados de monocitos extraídos de sangre periférica de donantes. La activación de monocitos fue determinada mediante citometría de flujo, reacción en cadena de polimerasa reversa y ELISA. La expresión de marcadores relacionados con el proceso inflamatorio en macrófagos fue determinada mediante citometría de flujo. La dieta elaborada con AOVE disminuyó significativamente la producción de oxido nítrico (NO) y citoquinas en

macrófagas peritoneales procedentes de ratones con LES. La FP del AOVE disminuyó la producción de NO y citoquinas inflamatorias junto con la expresión génica y proteica de marcadores inmunoinflamatorios en monocitos humanos. Además, la FP moduló la expresión de marcadores anti y proinflamatorios en macrófagos humanos. Estos resultados ponen de manifiesto el papel beneficioso del consumo de AOVE, como un alimento funcional de primera línea, y el especial protagonismo de la FP en dichos efectos beneficiosos, pudiendo ejercer efectos preventivos y moduladores en el tratamiento de enfermedades inmunoinflamatorias, como el LES.

POTENTIAL INVOLVEMENT OF THE PHENOLIC FRACTION OF EXTRA VIRGIN OLIVE OIL IN PRISTANE-INDUCED LUPUS IN MICE BY REGULATING PPAR-γ DEPENDENT MONOCYTE ACTIVATION

Marina Aparicio-Soto[1], Sergio Montserrat de la Paz[2], Marina Sánchez-Hidalgo[1], Ana Cárdeno[1], Beatriz Bermúdez[2], Francisco J. Garcia Muriana[2], Catalina Alarcón-de-la-Lastra[1]

[1]Department of Pharmacology, Faculty of Pharmacy, University of Seville, Spain
[2]Laboratory of Cellular and Molecular Nutrition, Instituto de la Grasa, CSIC, Seville, Spain.

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ABSTRACT

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by a loss of balance control of cellular immunoregulation. Monocytes and macrophages are critical effectors and regulators of inflammation and innate immune response. Recent studies suggested that extra virgin olive oil (EVOO) might possess preventive effects on immune-inflammatory diseases management. The present work evaluates the impact of an EVOO diet on peritoneal macrophages from pristane-induced SLE model mice as well as the effect of the EVOO Phenolic fraction (PE) on human monocytes and monocyte-derived macrophages. BALB/c mice received a pristane or saline solution injection and were fed with sunflower oil diet or EVOO diet. After 24 weeks, mice were sacrificed and peritoneal macrophages cultured to evaluate inflammatory markers. *In vitro* studies with primary human monocytes and monocyte-derived macrophages were performed. Monocyte and macrophage activations were measured by flow cytometry and diverse parameters implicated in the inflammatory process were determined by reverse transcription polymerase chain reaction (RT-qPCR) and Enzyme-Linked ImmunoSorbent Assay (ELISA). EVOO diet significantly reduced nitric oxide (NO) and inflammatory cytokines on peritoneal macrophages from SLE mice. PE decreased NO, cytokine production together with gene and protein expression of immune-inflammatory markers on human monocytes. Moreover, PE treatment downregulated the gene expression of M1 markers and up regulated M2 molecules on M0 macrophages. Altogether, our findings outline that EVOO

and particularly its PE have a significant therapeutic potential for the treatment of immune-inflammatory diseases such as SLE, through the modulation of monocytes/macrophages over-activation, decreasing NO and cytokine production as well as several immune-inflammatory markers, thereby offering a new promising therapeutic strategy in SLE management whose clinical use in SLE patients needs to be further investigated.

Keywords: EVOO, SLE, immunomodulation, macrophages, monocytes.

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CHAPTER III

THE PHENOLIC FRACTION OF EXTRA VIRGIN OLIVE OIL MODULATES THE ACTIVATION AND THE INFLAMMATORY RESPONSE OF T CELLS FROM PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS AND HEALTHY DONORS

LA FRACCIÓN POLIFENÓLICA DEL ACEITE DE OLIVA VIRGEN EXTRA MODULA EL PROCESO INFLAMATORIO EN CÉLULAS T DE PACIENTES CON LUPUS ERITEMATOSO SISTÉMICO E INDIVIDUOS SANOS

Marina Aparicio-Soto[1], Marina Sánchez-Hidalgo[1], Ana Cárdeno[1], José Manuel Lucena[2], Francisca Gonzalez-Escribano[2], María Jesús Castillo Palma[3], Catalina Alarcón-de-la-Lastra[1]

[1] Departamento de Farmacología, Facultad de Farmacia, Universidad de Sevilla, España.
[2] Departamento de Inmunología, Instituto de biomedicina (IBIS), Universidad de Sevilla, España, Hospital universitario Virgen del Rocío, Sevilla, España
[3] Unidad de colagenosis e hipertensión pulmonar Hospital universitario Virgen del Rocío, Sevilla, España

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RESUMEN

El lupus eritematoso sistémico (LES) es una enfermedad crónica multisistémica caracterizada por una desregulación del sistema inmune, durante la cual se produce una respuesta anómala de las células T y un desbalance en la producción de citoquinas. La fracción polifenólica (FP) del aceite de oliva virgen extra (AOVE) posee propiedades antiinflamatorias e inmunomoduladoras y ha demostrado efectos preventivos en modelos murinos de enfermedades inmunoinflamatorias, como es el caso del LES. En el presente estudio se evalúaron los efectos de la FP del EVOO en células mononucleares periféricas de pacientes con LES e individuos sanos. El fenotipo de las células T fue investigado mediante citometría de flujo, los niveles de citoquinas fueron determinados por ELISA y la expresión de diversas proteínas implicadas en el proceso inflamatorio fue determinada por Western Blot. La FP disminuyó la frecuencia de células CD69+ y la producción de interferón γ, factor de necrosis tumoral e interleucina (IL)-6, IL-1β e IL-10. Además, la FP incrementó la expresión de la proteína inhibitoria I-kappa-B y disminuyó la fosforilación de la proteína ERK en pacientes con LES y pacientes sanos. En base a estos resultados podemos afirmar que la FP modula la producción de citoquinas inflamatorias y la activación de células T probablemente a través de la vía del factor nuclear κB, poniendo

de manifiesto los efectos antiinflamatorios e inmunomoduladores de la FP y señalando su posible interés como una interesante alternativa en el tratamiento nutricional del LES.

THE PHENOLIC FRACTION OF EXTRA VIRGIN OLIVE OIL MODULATES THE ACTIVATION AND THE INFLAMMATORY RESPONSE OF T CELLS FROM PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS AND HEALTHY DONORS

Marina Aparicio-Soto[1], Marina Sánchez-Hidalgo[1], Ana Cárdeno[1], Jose Manuel Lucena[2], Francisca Gonzalez-Escribano[2], Maria Jesus Castillo Palma[3], Catalina Alarcón-de-la-Lastra[1]

[1] Department of Pharmacology, Faculty of Pharmacy, University of Seville, Spain

[2] Department of Immunology, Biomedicine Institute of Seville (IBIS), University of Seville, Virgen del Rocío, University Hospital, Seville, Spain

[3] Collagenosis and Pulmonary Hypertension Unit, Virgen del Rocío University Hospital, Seville, Spain

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M. Aparicio-Soto, M. Sánchez-Hidalgo, A. Cárdeno, J.M. Lucena, F. Gonzalez-Escribano, M.J. Castillo, C. Alarcón-de-la-Lastra. The phenolic fraction of extra virgin olive oil modulates the activation and the inflammatory response of T cells from patients with systemic lupus erythematosus and healthy donors.

ABSTRACT

Systemic lupus erythematosus (SLE) is a chronic multiorgan autoimmune disease characterized by immune deregulation, which involves altered T-cell response and imbalance of cytokine production. The phenolic fraction (PE) of extra virgin olive oil (EVOO) possesses anti-inflammatory and immunomodulatory properties and exerts preventive effects in murine models of immunoinflammatory diseases, such as SLE. The present study was designed to determine the *in vitro* effects of the PE from EVOO on peripheral blood mononuclear cells (PBMC) from patients with SLE and healthy donors. T cell phenotype was investigated by flow cytometry, cytokine levels were determined by ELISA and protein expression was detected by Western Blot. The PE of EVOO decreased the frequency of CD69+cells and the secretion of interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β and IL-10. Moreover, PE increased the expression of I-kappa-B (IκB)-α and decreased ERK phosphorylation on PBMC from patients with SLE and healthy donors. PE modulates cytokine production and attenuates induced T cell activation, probably through nuclear factor (NF-κB) signaling pathway, providing the first evidence that PE from EVOO has an anti-inflammatory and immunomodulatory role in SLE patients and it might therefore be considered as a dietary complement in SLE management

Keywords: EVOO, immunomodulation, olive oil, SLE, PBMC

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CHAPTER IV

NATURALLY OCCURRING HYDROXYTYROSOL DERIVATIVES: HYDROXYTYROSYL ACETATE AND 3,4-DIHYDROXYPHENYLGLYCOL MODULATE INFLAMMATORY RESPONSE IN MURINE PERITONEAL MACROPHAGES. POTENTIAL UTILITY AS NEW DIETARY SUPPLEMENTS.

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COMPUESTOS POLIFENÓLICOS DERIVADOS DEL HIDROXITIROSOL PRESENTES EN EL ACEITE DE OLIVA VIRGEN EXTRA MODULAN LA RESPUESTA INFLAMATORIA EN MACRÓFAGOS PERITONEALES MURINOS. POTENCIAL USO COMO SUPLEMENTOS DIETÉTICOS.

Marina Aparicio-Soto[1], Susana Sánchez-Fidalgo[1], Alejandro González-Benjumea[2], Inés Maya[2], José G. Fernández-Bolaños[2], Catalina Alarcón-de-la-Lastra[1].

[1] Departamento de Farmacología, Facultad de Farmacia, Universidad de Sevilla, España
[2] Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, España

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M. Aparicio-Soto, S. Sánchez-Fidalgo, A. Gonzalez-Benjumea, I. Maya, J. Fernández-Bolaños, C. Alarcón-de-la-Lastra. Naturally occurring hydroxytyrosol derivatives: hydroxytyrosyl acetate and 3,4-dihydroxyphenylglycol modulate inflammatory response in murine peritoneal macrophages. Potential utility as new dietary supplements. J Agric Food Chem. (2015). 63, 836-846.

RESUMEN

En el presente trabajo se evalúaron los efectos de dos compuestos polifenólicos presentes en el aceite de olive virgen extra (AOVE): hidroxitirosil acetato (2) y 3,4-dihidroxifenilglicol (3), así como dos nuevos acil derivados de 3: 4-(1',2'-dibutanoiloxietil)benceno-1,2-diol (7) y 4-(1',2'-dilauroiloxietil)benceno-1,2-diol (8), en comparación con hidroxitirosol (HTy, 1), en un modelo de inflamación en macrófagos peritoneales murinos estimulados con lipopolisácarido bacteriano (LPS). Los compuestos 2, 3, 7 y 8 mostraron una marcada actividad antioxidante, previniendo la formación de especies reactivas de oxigeno y disminuyendo los niveles de nitritos y la expresión de la enzima oxido nítrico sintasa inducible (iNOS). Sin embargo, solo 2 y 3 disminuyeron la expresión de la enzima ciclooxigenasa inducible (COX-2) mientras que los derivados diacetilados 7 y 8 demostraron una menor actividad antiinflamatoria que el compuesto original 3. En conclusión, los compuestos fenólicos 2 y 3 presentan una marcada actividad antiinflamatoria en un modelo de inflamación en macrófagos peritoneales murinos, pudiendo contribuir a los efectos beneficiosos atribuidos al consumo de AOVE y constituyendo una interesante alternativa al hIdroxItIrosol. debido a su mejor perfil farmacocinético y farmacodinámico.

NATURALLY OCCURRING HYDROXYTYROSOL DERIVATIVES: HYDROXYTYROSYL ACETATE AND 3,4-DIHYDROXYPHENYLGLYCOL MODULATE INFLAMMATORY RESPONSE IN MURINE PERITONEAL MACROPHAGES. POTENTIAL UTILITY AS NEW DIETARY SUPPLEMENTS

Marina Aparicio-Soto[1], Susana Sánchez-Fidalgo[1], Alejandro González-Benjumea[2], Inés Maya[2], José G. Fernández-Bolaños[2], Catalina Alarcón-de-la-Lastra[1].

[1] Department of Pharmacology, Faculty of Pharmacy, University of Seville, Spain

[2] Department of Organic Chemistry, Faculty of Chemistry, University of Seville, Spain

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ABSTRACT

This work evaluated the effects of extra virgin olive oil (EVOO) phenols, hydroxytyrosyl acetate (2) and 3,4-dihydroxyphenylglycol (3), as well as two new acyl derivatives of 3, 4-(1,2-di(butanoyloxy)ethyl)benzene-1,2-diol (7) and 4-(1,2-di(lauroyloxy)ethyl)benzene-1,2-diol (8), on LPS-stimulated murine peritoneal macrophages in comparison with hydroxytyrosol (HTy, 1). Compounds 2, 3, 7 and 8 showed a strong reactive oxygen species (ROS)-scavenging activity, reducing significantly nitrites levels with a significant decrease on iNOS expression [2 (50 μM, 0.44±0.03; 100 μM, 0.44±0.01; $p<0.01$); 3 (50 μM, 0.37±0.03; 100 μM, 0.37±0.01; $p<0.001$); 7 (50 μM, 0.45±0.06; $p<0.01$)]. However, only 2 and 3 downregulated COX-2 expression [2 (50 μM, 0.72±0.04, $p<0.05$; 100 μM, 0.54±0.06, $p<0.01$); 3 (50 μM, 0.56±0.05, $p<0.05$; 100 μM, 0.37±0.04; $p<0.001$)] and prevented IKBα degradation [2 (100 μM, 1.63±0.14, $p<0.01$); 3 (100 μM, 1.82±0.09; $p<0.01$)]; the diacylated compounds 7 and 8 showing worse anti-inflammatory activity than the parent 3. In conclusion, 2 and 3 phenolic derivatives could play an important role in the anti-inflammatory effect of EVOO. The implication of this study for nutrition and general health of the population rests in the possible use of natural HTy derivatives with better hydrophilic/lipophilic balance, thus improving its pharmacodynamic and pharmacokinetic profiles, as new dietary supplements in foods.

Keywords: EVOO, phenols, inflammation, macrophages, NF-κB.

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Chapter IV

CHAPTER V

DIETARY HYDROXYTYROSOL AND HYDROXYTYROSYL ACETATE SUPPLEMENTATION PREVENT PRISTANE-INDUCED SYSTEMIC LUPUS ERYTHEMATOUS IN MICE

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EFECTO PREVENTIVO DE DIETAS ELABORADAS CON HIDROXITIROSOL Y HIDROXITIROSIL ACETATO, POLIFENOLES PRESENTES EN EL ACEITE DE OLIVA VIRGEN EXTRA, EN UNA MODELO MURINO DE LUPUS ERITEMATOSO SISTÉMICO INDUCIDO POR PRISTANO

Marina Aparicio-Soto[1], Marina Sánchez-Hidalgo[1], Ana Cárdeno[1], Alejandro González-Benjumea[2], José G. Fernández-Bolaños[2], Catalina Alarcón-de-la-Lastra[1]

[1] Departamento de Farmacología, Facultad de Farmacia, Universidad de Sevilla, España
[2] Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, España

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RESUMEN

Recientes estudios han puesto de manifiesto que diversos compuestos polifenólicos presentes en la dieta pueden tener un papel beneficioso en la prevención y manejo de enfermedades inmunoinflamatorias. El lupus eritematoso sistémico (LES) es una enfermedad autoinmune inflamatoria crónica para la que actualmente no se dispone de un tratamiento efectivo y seguro. Por lo tanto, la terapia nutricional puede constituir una interesante alternativa para el tratamiento del LES, debido a sus potenciales beneficios sin los efectos adversos asociados a la farmacoterapia clásica. En este estudio evaluamos los efectos de dos dietas suplementadas con polifenoles del aceite de oliva virgen extra: hidroxitirosol (HTy) y acetato de hidroxitirosol (HTy-Ac) en un modelo de LES inducido por pristano en ratones BALB/c. Veinticuatro semanas tras la inducción del LES, esplenocitos y macrófagos peritoneales fueron extraídos y cultivados para evaluar citoquinas pro y antiinflamatorias. Los cambios en la expresión de proteínas renales fueron determinados por Western blot. Nuestros resultados nos permiten concluir que la suplementación dietética con HTy o HTy-Ac reduce significativamente la producción de citoquinas inflamatorias en riñón, macrófagos peritoneales y esplenocitos y previene el daño renal inducido por pristano, disminuyendo la expresión de diversas proteínas implicadas en el

daño renal través de diversas vías implicadas en el LES, sugiriendo que ambos compuestos fenólicos pueden representar una nueva alternativa para el abordaje dietético del LES.

DIETARY HYDROXYTYROSOL AND HYDROXYTYROSYL ACETATE SUPPLEMENTATION PREVENT PRISTANE-INDUCED SYSTEMIC LUPUS ERYTHEMATOUS IN MICE

Marina Aparicio-Soto[1], Marina Sánchez-Hidalgo[1], Ana Cárdeno[1], Alejandro González-Benjumea[2,] José G. Fernández-Bolaños[2], Catalina Alarcón-de-la-Lastra[1]

[1] Department of Pharmacology, Faculty of Pharmacy, University of Seville, Spain

[2] Department of Organic Chemistry, Faculty of Chemistry, University of Seville, Spain

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ABSTRACT

Current experimental studies suggest a beneficial role of dietary phenolic compounds on prevention and management of immune-inflammatory disorders. Systemic lupus erythematosus (SLE), a multisystemic autoimmune chronic disease, remains without an effective and safe treatment. Thus, diet therapy could be a promising approach in SLE due to its potential prophylactic effects without the side effects of classical pharmacology. This study evaluates the effects of diets supplemented with phenolic compounds: hydroxytyrosol (HTy) and hydroxytyrosyl acetate (HTy-Ac) in pristane-induced SLE mice. Peritoneal macrophages and spleens were isolated and cultured to evaluate cytokines levels by Enzyme-Linked Immunosorbent Assay (ELISA). Renal changes of inflammatory markers and signaling pathways were determined by western blot. Dietary phenol supplementation significantly reduced proinflammatory cytokines and prevented renal damage with a considerably blockage of different inflammatory-related pathways suggesting that HTy and HTy-Ac supplementation might provide a basis for developing a new dietary strategy for prevention and management of SLE.

Keywords: diet, immunomodulation, inflammation, lupus, phenols, SLE

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CHAPTER VI

OLIVE OIL-DERIVED PHENOLS EXERT EFFECTIVE ANTI-INFLAMMATORY PROPERTIES BY INTERFERENCE WITH THE NF-κB PATHWAY AND TSLP PRODUCTION IN HUMAN KERATINOCYTES

COMPUESTOS FENÓLICOS DEL ACEITE DE OLIVA VIRGEN EXTRA MODULAN LA RESPUESTA INFLAMATORIA A TRAVÉS DE LA VÍA DEL NF-κB EN QUERATINOCITOS HUMANOS

Marina Aparicio-Soto[1], Margitta Worm[2], Marina Sánchez-Hidalgo[1], José G. Fernández-Bolaños[3], Catalina Alarcón-de-la-Lastra[1], Magda Babina[2]

[1]Departamento de Farmacología, Facultad de Farmacia, Universidad de Sevilla, España
[2]Departamento de Dermatología y Alergología, Clínica Universitaria Charité, Berlín, Alemania
[3]Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, España

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RESUMEN

El aceite de oliva virgen extra (AOVE) es rico en una serie de compuestos fenólicos, incluyendo hidroxitirosol (HTy) y acetato de hidroxitirosol (HTy-Ac), que han presentado un amplio rango de efectos beneficiosos. Sin embargo, los posibles beneficios de estos compuestos fenólicos no han sido aún estudiados en el proceso inflamatorio de la piel. El presente estudio fue diseñado para evaluar los potenciales efectos de HTy y HTy-Ac en un modelo de inflamación *in vitro* con queratinocitos humanos y esclarecer los mediadores y vías de señalización implicados. Para ello, queratinocitos humanos fueron obtenidos de piel de individuos sanos y pre-tratados con HTy-Ac o HTy. Las células fueron estimuladas con interleucina (IL)-1β o con ligando de receptor de tipo Toll 3 (TR3-l) a distintos tiempos experimentales. Los niveles de la citoquina linfopoyetina estromal tímica (TSLP) fueron evaluados por ELISA, mientras que los niveles de expresión génica de TSLP, sus isoformas y diversos genes proinflamatorios fueron determinados mediante la reacción en cadena de la polimerasa con transcriptasa inversa (RT-qPCR). La expresión del factor nuclear kappa B (NF-κB) fue estudiada mediante Western blot. Nuestro estudio puso de manifiesto que HTy y HTy-Ac disminuyeron significativamente la producción y expresión génica de TSLP, junto con los niveles de otros genes proinflamatorios (TNF-α, IL-6, IL-8 y COX-2) en queratinocitos

humanos, posiblemente a través de la modulación de la actividad de la vía del NF-κB, mediante de la prevención de la degradación de la proteína inhibitoria IκBα y la inhibición de la translocación nuclear de la proteína p65. Estos resultados nos permiten concluir que ambos compuestos fenólicos mejoran el balance inflamatorio en este modelo de queratinocitos humanos a través de la regulación de la producción y expresión génica de TSLP y otros mediadores inflamatorios mediante la modulación de la vía del NF-κB, pudiendo constituir una nueva alternativa para el abordaje de enfermedades inflamatorias de la piel, como es el caso del LES.

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Marina Aparicio-Soto[1], Margitta Worm[2], Marina Sánchez-Hidalgo[1], José G. Fernández-Bolaños[3], Catalina Alarcón-de-la-Lastra[1] ,Magda Babina[2]

[1]Department of Pharmacology, Faculty of Pharmacy, University of Seville, Spain
[2]Allergy Center Charité, CCM, Department of Dermatology and Allergy, Charité-Universitätsmedizin Berlin, Germany
[3] Department of Organic Chemistry, Faculty of Chemistry, University of Seville, Spain

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Marina Aparicio-Soto, Margitta Worm, Marina Sánchez-Hidalgo, José G. Fernández-Bolaños, Catalina Alarcón-de-la-Lastra, Magda Babina. Olive oil-derived phenols exert effective anti-inflammatory properties by interference with the NF-κB pathway and tslp production in human keratinocytes.

ABSTRACT

Extra virgin olive oil (EVOO) is rich in diverse phenolic compounds, including hydroxytyrosol (HTy) and hydroxytyrosyl acetate (HTy-Ac) that have present a wide range of beneficial properties. However, the potential benefit from these EVOO bioactive compounds in the inflammatory balance of the skin have not yet been elucidated. The present study was designed to evaluate the potential effects of HTy and HTy-Ac in an *in vitro* model of human keratinocytes and clarify the mediators and molecular mechanism involved. Human keratinocytes were isolated from skin of healthy donors. HTy-Ac or HTy were added at concentrations ranging 12.5 to 100 μM and 30 minutes later, cells were stimulated with interleukin (IL)-1β or Toll-like receptor 3 ligand (TR3-l) for different time points. Levels of thymic stromal lymphopoietin (TSLP) were evaluated by enzyme-linked immunosorbent assay. TSLP and TSLP isoforms mRNA levels as well as other genes related to the inflammatory response were evaluated by quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR). The expression of nuclear transcription factor-kappa B (NF-κB) pathway was determined by Western blot. EVOO phenols significantly reduced TSLP production and TSLP and its isoforms mRNA levels, as well as proinflammatory gene levels (TNF-α, IL-6 and IL-8) in human keratinocytes. Moreover, HTy and HTy-Ac counteracted induced NF-κB activity through prevention of IκBα degradation and p65 nuclear

translocation. Thus, these EVOO polyphenols, HTy-Ac and HTy, efficiently improve the inflammatory process in human keratinocytes by decreasing TSLP and other inflammatory mediators through interference with the NF-κB pathway.

Keywords: EVOO, inflammation, olive oil, skin, TSLP

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RESULTS
&
GENERAL DISCUSSION

The focus of the **chapter I** was to elucidate the potential protective role of a diet elaborated with extra virgin olive oil (EVOO) in the prevention and development of systemic lupus erythematosus (SLE) in a murine pristane-induced experimental lupus model. The results of the present work indicate, for the first time, that dietary EVOO effectively had a preventive and therapeutic role in the development of SLE in female BALB/c mice pristane-induced model of SLE in comparison with those pristane mice fed with SD diet. This beneficial effect was well correlated to an improved renal damage score, as reflected by milder proteinuria, reduced paw swelling **(Chapter I. Figure 1A)** and a minor inflammatory mononuclear cells infiltration in renal interstitium **(Chapter I. Figure 2).**

Imbalance of Th subsets (Th1/Th2/Th17) and regulatory T cells (Tregs) is suggested to contribute to the pathogenesis of SLE that leads to loss of self-tolerance with activation of autoreactive T and B cells thereby a production of autoantibodies that contributes to deposition of immune complexes leading to the production of large amounts of inflammatory cytokines that contribute actively to local inflammation and tissue damage (Shlomchik et al., 2001; Talaat et al., 2015). Our results revealed that dietary EVOO significantly reduced tumour necrosis factor (TNF)-α, interleukin (IL)-6, IL-10 and IL-17 production in lipopolysaccharide (LPS)-stimulated splenocytes from pristane-induced lupus mice in comparison with those animals fed with sunflower diet (SD) **(Chapter I. Figure 8).** In this sense, the EVOO-mediated suppression of LPS-induced inflammatory cytokines may also be beneficial for attenuating pristane-induced lupus glomerulonephritis.

Metalloproteinases (MMP) act on proinflammatory cytokines, chemokines and other proteins regulating varied aspects of inflammation and immunity. Particularly, MMP-3 levels have shown to be significantly higher in SLE patients and associated with arthritis, nephritis and haematological manifestations with a history of persistent proteinuria, anti-ds-DNA antibodies, circulating immune complexes and decreased complement C levels (Kotajima et al., 1998). According to our data, MMP-3 serum levels increased significantly in pristane-treated mice fed with SD diet whereas those pristane-treated animals which received EVOO diet expressed similar serum MMP-3 levels to those described in control mice **(Chapter I. Figure 4A)**, contributing to minimize renal damage produced by pristane induction.

Mitogen-activated protein kinases (MAPK) family members, including p38 kinases, ERK1 and ERK2 and JNKs, are involved in many important cell processes, mainly the regulation of the synthesis of chemokines, cytokines, adhesion molecules and prostaglandins (PG) involved in the regulation of cellular and humoral autoimmune responses (Thalhamer et al., 2008). Moreover, MAPK phosphorylate the janus kinase- signal

transducer and activator of transcription (JAK/STAT) that is important in proinflammatory cytokine-mediated signaling pathways such as Th17 cell differentiation, including IL-17A, promoting effector T cell phenotypes (Korn et al., 2009) leading to STAT3 activation by phosphorylation on tyrosine residues resulting in the formation of STAT dimers that translocate into the nucleus to bind specific DNA sequences(Aaronson and Horvath, 2002). We demonstrated, for the first time, that pristane injection increased kidney phosphorylation of p38, JNK and ERK MAPK in those animals fed with SD. STAT3 overexpression was also evidenced in kidney from SD-pristane mice and was positively related to the severity of renal damage whereas EVOO diet intake reduced significantly both MAPK and STAT3 activation at transcriptional level **(Chapter I. Figure 6B and 7)**. Altogether, our results suggest that dietary EVOO may repress IL-10 and IL-17 production interfering negatively with JNK, p38, pERK MAPK and STAT3 signaling pathways.

PGE_2 modulates a variety of immune processes at sites of inflammation, including production of inflammatory cytokines, and has been implicated in the glomerular filtration regulation (Imig et al., 2002). PGE_2 levels have been remarkably increased in kidney from pristane-induced mice in comparison with healthy control group (Chae et al., 2008). In addition, microsomal prostaglandin E synthase-1 (mPGES-1), a crucial enzyme in the PGE_2 biosynthesis, has been found in the renal collecting ducts, macula densa, and medullary interstitial cells (Schneider et al., 2004) and high urinary levels of this prostanoid have been reported in lupus nephritis(Herrera-Acosta et al., 1987). Our report is in agreement with these results, demonstrating that EVOO diet ameliorates PGE_2 production and mPGES-1 protein expression in kidney from pristane-treated mice **(Chapter I. Figure 4B).**

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a key transcription factor involved in the regulation of immune responses, has been demonstrated to be a potential candidate for studies concerning the pathogenesis of autoimmunity and chronic inflammatory diseases such as rheumatoid arthritis and SLE, among others (Karin, 2006; Makarov, 2001). Particularly, accumulating evidence has demonstrated the involvement of NF-κB in self-reactive T- and B-lymphocyte development, survival and proliferation, as well as the maintenance of chronic inflammation due to cytokines, including TNF-α, IL-1, 6 and IL-17. In our study, inhibitor of kappa B (IκBα) protein expression in kidney decreased after pristane injection in mice fed with SD. On the contrary, in those kidneys from pristane-treated mice fed with EVOO showed similar IκBα protein expression levels to those described in control group animals **(Chapter I. Figure 6A).**

The generation of reactive oxygen species (ROS) plays a pivotal role in both acute and chronic glomerular injuries in patients with lupus nephritis. The Nuclear factor

(erythroid-derived 2)-like 2 (Nrf2) is a key transcription factor modulating heme oxygenase (HO)-1 expression. In Nrf2-/- mice, a murine lupus nephritis model established using pristane injection, suffered from greater renal damage and had more severe pathological alterations in the kidney. On the contrary, Nrf2+/+ mice showed ameliorative renal function when treated with sulforaphane, an Nrf2 inducer (Jiang et al., 2014). Similarly, the administration of an HO-1 inducer reduced nephritis severity in the MRL-Fas lpr mouse model of lupus (Takeda et al., 2004). Importantly, patients with SLE display a decreased expression of HO-1 on peripheral blood monocytes, which might be related to SLE pathogenesis, suggesting that HO-1 induction could be considered a potential therapy to improve SLE progression(Herrada et al., 2012). Additionally, serum HO-1 levels have been shown to be high in patients with SLE but decreased in those with proliferative lupus nephritis, where these levels were associated with poor prognosis. In our study, dietary EVOO strongly activated HO-1/Nrf2 antioxidant pathway contributing to the beneficial effects of EVOO in this murine model of chronic inflammation **(Chapter I. Figure 5),** suggesting that Nrf2 may protect against lupus nephritis by inhibiting the activation of the NF-κB pathway and deposition of extracellular matrix.

Since our results show that EVOO diet was more effective in reducing SLE severity in comparison with SD diet, we could suggest that the responsibility for such beneficial properties could to be assigned to both an adequate fatty acid profile of EVOO and the high proportion of phenolic compounds in according with our recent data (Aparicio-Soto et al., 2015; Rosillo et al., 2014; Sánchez-Fidalgo et al., 2015a). Also the presence of other valuable minor components present in the unsaponifiable fraction of the oil such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, phytosterols with important anti-inflammatory properties (Cardeno et al., 2014a; Cárdeno et al., 2014; Sánchez-Fidalgo et al., 2015b) could play a pivotal role. Moreover, these improved effects observed could be due to a possible synergistic effect among EVOO constituents, since it is not clear whether all the possible beneficial mechanisms act independently of each other or whether they have a synergistic or competitive action (Alarcon de la Lastra Romero, 2011).

In summary, our findings suggest that dietary EVOO has protective effects for alleviating pristane-induced lupus in mice reducing the levels of inflammatory mediators and activating HO-1/Nrf2 antioxidant pathway by blocking JAK/STAT, NF-κB and JNK/p38 MAPK-mediated inflammatory response and may constitute an important functional food and a novel approach for managing SLE. Nevertheless, additional studies in other lupus models and well-designed clinical trials are needed to confirm these findings and explore its therapeutic effects in humans.

In the **chapter II**, we evaluated the impact of EVOO diet on peritoneal macrophages from pristane-induced SLE model mice as well as the effect of the EVOO Phenolic fraction (PE) on human monocytes and monocyte-derived macrophages

In the past, most of SLE pathogenesis studies considered the adaptive immune system to be the primary cause of autoimmunity, focusing on the primary abnormalities of B and T lymphocyte functions. However, nowadays studies are also centered on the innate immunity and on how the SLE autoimmune response is initiated and maintained (Li et al., 2010). In this sense, monocytes and macrophages play a pivotal role in the innate immune system with widespread immunological function (Unanue, 1978) and abnormalities in it phenotype and functions have been associated with a variety of autoimmune disorders, including SLE (Katsiari et al., 2010).

Oxidative stress plays a substantial role not only in the pathogenesis of autoimmune rheumatic diseases and their complications, but also on specific disease activity. Particularly, a deregulation of redox homeostasis may lead to overproduction of proinflammatory cytokines and nitric oxide (NO) leading to a condition of oxidative stress which plays an important role in SLE (Sukkar and Rossi, 2004). Furthermore, several lines of evidence suggested mechanisms through which the activity of inducible nitric oxide synthase (iNOS) is pathogenic on SLE, altering enzyme activity, increasing the immunogenicity of self antigens, modifying immune tolerance and leading to glomerular and vascular pathology (Oates and Gilkeson, 2006). Thus, iNOS and control oxidative stress targeted therapies may provide the means to reduce the pathogenic consequences of SLE. In this regard, we have shown that EVOO diet decreased NO production on LPS-stimulated peritoneal macrophages from pristane induced BALB/c mice **(Chapter II. Figure 1A)**. Additionally, PE treatment significantly reduced NO with a significant decrease of iNOS protein and NOS-2 gene expression on stimulated human monocytes **(Chapter II. Figure 2A, 2B and 2C)**. These findings are in accordance with those previously described on LPS-activated murine macrophages and acute experimental colitis in mice, where PE inhibited LPS-induced iNOS protein expression through NF-κB and MAPK (Cardeno et al., 2014b; Sanchez-Fidalgo et al., 2013).

Modulation of proinflammatory mediators in macrophages is considered one of the strategies to develop therapeutic compounds against several inflammatory diseases. In this regard, Toll like receptor (TLR)-4 plays important roles in the process of macrophage activation and polarization and its activation by LPS triggers the release of cytokines such as TNF-α, IL-1β and IL-6 (Chen et al., 2015). Our results revealed that dietary EVOO significantly reduced TNF-α, IL-6 and IL-17 production on LPS-stimulated peritoneal

macrophages from pristane-induced lupus mice in comparison with those animals fed with SD suggesting that this EVOO-mediated suppression of cytokines may be beneficial for attenuating pristane-induced lupus **(Chapter II. Figure 1B)**. Moreover, PE decreased TLR4 mRNA levels as well as the gene expression of TNF-α, IL-1β and IL-6 proinflammatory cytokines on LPS-activated human monocytes **(Chapter II. Figure 3)**.

Despite the limited available data related to peroxisome proliferator-activated receptors (PPAR)-γ and SLE, some studies have shown the possible therapeutic benefits of PPAR-γ agonists on this disease (Venegas-Pont et al., 2009). Thus, we further studied PPAR-γ signaling pathway, which activation inhibits the expression of inflammatory cytokines and directs the differentiation of immune cells towards anti-inflammatory phenotypes (Chinetti et al., 2000; Martin, 2009). According to our results, human monocytes were responsive to LPS stimulation by decreasing PPAR-γ levels, whereas PE treatment was able to significantly increased PPAR-γ protein expression as well as PPAR-γ mRNA levels on human monocytes suggesting a possible modulation of macrophage activation and polarization by PE EVOO **(Chapter II. Figure 2D and 2E)**.

Macrophages are key modulator and effector cells in the immune response because their activation influences and responds to other arms of the immune system. In this regard, macrophages can be classified into M0, M1 and M2 subsets: M1 macrophages [activated by LPS or interferon (IFN)-γ] express a spectrum of proinflammatory molecules, such as IL-1β, IL-6, NO and iNOS whereas M2 macrophages (activated by IL-4) express a wide array of anti-inflammatory molecules (Perez-Jimenez et al., 2005). Our results showed that LPS and IFN-γ increased the expression of M1 markers on M0 macrophages whereas IL-4 up regulated the gene expression of M2 markers. On the contrary, PE treatment downregulated the gene expression of M1 markers and up regulated M2 molecules on M0 macrophages suggesting that the PE from EVOO prevents the classical macrophages activation by LPS and IFN-γ regulating inflammatory cytokine production and polarized M0 macrophages into M2 macrophages contributing to its anti-inflammatory activities **(Chapter II. Figure 4)**.

Finally, we explored whether PE could modify monocyte subset distribution. Human monocytes can be classified into two general categories based on their expression of cell-surface markers. Most monocytes are CD14++CD16+, which are referred as "classical" monocytes and considered as proinflammatory cells because after LPS stimulation they exhibit IL-6 and IL-10 profile and produce high levels of C-C chemokine receptor type 2 (CCR2), which promotes monocyte migration through inflammation sites. "Non-classical" monocytes (CD14+ CD16++) monocytes express low levels of CD14 (a co-receptor with

TLR4 for LPS) resulting in a lower cytokine and chemokine response to LPS as well as lower levels of CCR2, decreasing their ability to migrate in response to inflammatory stimuli (Moser et al., 2009; Yang et al., 2014). Moreover, "non-classical" monocytes have also modulatory effects on '"classical" monocytes and T cells and appear to be decreased and non-functional in SLE patients (Burbano et al., 2014). In this sense, PE caused a significant decrease of "classical" monocytes but a significant increase of "non-classical" monocytes on LPS human monocytes. Furthermore, PE treatment produced a significant reduction of CCR2+ cells and CCR2 gene expression levels on LPS-treated classical monocytes **(Chapter II. Figure 5)**.

Altogether, our findings outline that EVOO and particularly its PE might have a significant therapeutic potential for the treatment of immune-inflammatory diseases such as SLE, through the modulation of monocytes/macrophages over-activation, decreasing NO and cytokine production as well as several immune-inflammatory markers, thereby offering a new promising therapeutic strategy in SLE management whose clinical use in SLE patients needs to be further investigated.

In the **chapter III**, we determined the *in vitro* effects of the PE from EVOO on peripheral blood mononuclear cells (PBMC) from patients with SLE and healthy donors.

Several elements of the immune system are potential targets for therapeutic intervention in patients with SLE. In this sense, IL-6 is implicated in the development and progression of SLE in mice and it is identified as one of the major genetic risk factors for SLE in humans (Jain et al., 2016), thus its modulation seems to be a potential therapeutic approach in SLE management (Wallace et al., 2016). In the present study, we found a remarkable increase of IL-6 production in patients with SLE compared with healthy subjects. Moreover, IL-6 levels were significantly increased in both groups after phytohaemagglutinin (PHA) stimulation, whereas PE treatment was able to prevent this induced cytokine production **(Chapter III. Figure 1A)**.

In addition, some studies have provided evidence for the role of IFN in SLE pathogenesis (Hron and Peng, 2004; Santiago-Raber et al., 2003). Similarly, IFN levels are also directly correlated with SLE disease activity and it appears increased in sera from patients with SLE (Bakshi et al., 2015). Our study demonstrates that the *in vitro* treatment with PE positively regulated the induced production of IFN-γ on stimulated PBMC from patients with SLE and healthy donors, contributing to equilibrate this induced immune-inflammatory imbalance **(Chapter III. Figure 1B)**. In this context, studies on lupus prone mice and SLE patients have documented that high concentrations of TNF-α are correlated with the severity of kidney damage, SLE disease activity and anti-dsDNA antibodies

production (Mao et al., 2014; Umare et al., 2014). In our study, there were significant differences in TNF- α production between patients with SLE and healthy donors: the levels of this proinflammatory cytokine appeared significantly increased in unstimulated and PHA-stimulated cells from patients with SLE compared with healthy donors. Moreover, PE treatment was able to decrease in a significant way this induced production in both groups **(Chapter III. Figure 1C)**. The overproduction of IL-1β is involved in the pathogenesis of SLE and other autoimmune diseases (Umare et al., 2014). In the present study we did not find differences in IL-1β production after PHA stimulation on PBMC, but these results are in accordance with previous studies, where PHA stimulation did not alter the production of this cytokine (De Groote et al., 1992; Le Meur et al., 1999). However, we found that IL-1β production in patients with SLE was significantly lower than in healthy donors, which may be due to the inactive cohort selected for this study. Remarkably, PE treatment was capable of decreasing the production of this cytokine in both groups **(Chapter III. Figure 1D)**.

IL-10 is an important immunoregulatory cytokine with pleiotropic effects, which plays a crucial role in immune-inflammatory diseases. Particularly, IL-10 inhibits T cell function by suppressing the expression of proinflammatory cytokines such as TNF-α, IL-1 or IL-6 (Ding et al., 1993). High IL-10 expression seems to have a causal role in SLE pathogenesis and development of lupus nephritis and its polymorphisms contribute, at least in part, to the genetics involved in SLE (Abdallah et al., 2016; Zhang et al., 2013). Our study shows that PHA produces a significant increase of IL-10 levels that was significantly elevated in patients with SLE when compared with healthy donors. Moreover, PE treatment showed distinctive activities between groups: in healthy donors, IL-10 levels were enhanced after PE treatment whereas in patients with SLE its levels were significantly decreased **(Chapter III. Figure 1E)**. Therefore, since several studies in SLE have shown that anti-IL-10 treatment can decrease disease activity in terms of clinical features and biologic markers, PE from EVOO could be a promising approach for SLE management (Abdallah et al., 2016).

T cell activation is a fundamental step for the onset of the adaptive immune response. In this regard, CD69 is thought to be among the earliest surface markers of T-cells activation, and its changes are correlated with SLE disease activity (Draborg et al., 2014). In fact, active SLE patients show increased CD69 expression levels in comparison with inactive patients and healthy subjects (Chavez-Rueda et al., 2005). In the present study, we describe that the PE from EVOO significantly decreased CD69 induced expression levels on CD4+ T lymphocytes from SLE patients, with a similar but dose-dependent effect on CD4+ T lymphocytes from healthy donors. Moreover, after PHA stimulation, CD69

expression levels were lower in patients with SLE when compared with healthy donors, which could be due to the influence of the pharmacological therapy on the expression of activation markers on CD4+ T cells **(Chapter III. Figure 4)**.

Current evidence suggests that a pathologic CD4+ subset characterized by impaired extracellular signal–regulated kinases (ERK) pathway signal contributes to SLE pathogenesis. In this sense, defective T cell pathway signaling occurs in SLE and it has been directly implicated in causing epigenetic abnormalities that result in lupus-like autoimmunity (Gorelik and Richardson, 2010). Moreover, different studies have shown that EVOO as well as some EVOO bioactive components are able to modulate ERK phosphorylation in different inflammatory models, including pristane induced SLE in mice (Aparicio-Soto et al., 2016; Rosillo et al., 2015). In our study, we found that ERK phosphorylation was significantly increased after PHA stimulation compared with non-stimulated cells in patients with SLE and healthy donors. In addition, PE acts as a key positive regulator of ERK1/2 phosphorylation, decreasing PHA-induced phosphorylation. On the contrary, previous studies have shown that ERK signaling pathway is decreased in T cells from SLE patients, been associated with an over-activation of T cells (Wang et al., 2016). Our data did not shown this expected decreased expression of ERK pathway, but similar results between patients with SLE and healthy donors, but these discrepancies might be due to the inactive condition of the cohort of patients along with their pharmacological treatment **(Chapter III. Figure 3A)**. Nevertheless, given that the position of ERK in this disease seems to be controversial, further studies are needed to clarify the role of this pathway in SLE.

NF-κB is a transcription factor widely accepted as a critical regulatory modulator of inflammation and innate/adaptive immunity and its inhibition can reduce the expression of many genes encoding key inflammatory mediators, such as IL-6, IL-10 and TNF-α (Tak and Firestein, 2001; Wang et al., 1995). The deregulation of NF-κB activation is considered to drive many human diseases and particularly, several studies suggested that NF-κB might play a prominent role in the onset and progression of SLE, been critically involved in B cell differentiation and autoantibodies production (Criscione and Pisetsky, 2003; Hu et al., 2015). In our study we found that the expression of IκBα, the inhibitory protein of NF-κB, was significantly increased by PE treatment after PHA stimulation in patients with SLE as well as in healthy donors, with similar results in both groups, suggesting that the activity of PE might be related to its capacity to modulate NF-κB activation and it may be an important mechanistic link to the ability of diet to offset immune deregulation **(Chapter III. Figure 2A and 2B)**.

Although this study can make preliminary evidences of the therapeutic mechanism of PE from EVOO in SLE, the impact of EVOO and its bioactive components in patients with SLE requires further study. Regardless, the present study proves that PE modulates cytokine production and attenuates induced activation of T cells in patients with SLE and healthy donors, probably through NF-κB signaling pathway. Moreover, we found statistically differences in the production of IL-6, TNF-α, and IL-10 in patients with SLE, whose levels were markedly higher after PHA stimulation, and in the levels of IL-1β, which production was significantly lower. Furthermore, PE showed an interesting immunomodulatory effect in IL-10 production, enhancing IL-10 levels in healthy donors and decreasing them in patients with SLE. Thus, this study does provide the first evidence that PE from EVOO has an anti-inflammatory and immunomodulatory role in human PBMC, contributing to modulate the cytokine imbalance in patients with SLE and it might therefore be considered as a potential dietary strategy in SLE management.

In the **chapter IV**, we evaluated the effects of two EVOO phenols, hydroxytyrosol acetate (HTy-Ac, 2) and 3,4-dihydroxyphenylglycol (3), as well as two new acyl derivatives of 3, on LPS-stimulated murine peritoneal macrophages in comparison with hydroxytyrosol (HTy, 1).

Balance disruption of the intracellular reduction-oxidation state has been observed in activated macrophages, which leads to oxidative stress characterized by a major shift in the cellular redox balance and usually accompanied by ROS-mediated damage (Brüne et al., 2013). Therefore, modulators of ROS production and ROS-induced signaling pathways, especially in macrophages, could represent potential targets for anti-inflammatory intervention (Li et al., 2012). Our findings have shown for the first time that these phenol compounds, mainly compounds 2 and 3, showed significant antiradical and antioxidant activities and thus prevented the progression of cellular damage induced by LPS acting as effective antioxidants **(Chapter IV. Figure 1).**

Stimulation of macrophages by LPS induces transcription of iNOS gene and generation of large amounts of NO. NO acts as an intracellular messenger, which modulates the formation of endogenous ROS including hydrogen peroxide, peroxynitrite, and other potential oxidants that orchestrate the inflammatory response (Li et al., 2012). In the present study we found that exposure of peritoneal macrophages to LPS resulted in a significant increase of nitrite/nitrate levels as a NO production indicator and an upregulation of iNOS protein expression. However, 1-3, and 7 compounds reduced significantly both nitrites production and iNOS expression, in contrast to 8, which showed a

lower reduction of NO production at 50 μM but no changes in LPS-induced iNOS expression **(Chapter IV. Figure 2 and 3).**

The cyclooxygenase (COX)-2, the inducible isoform of COX, is the key enzyme that catalyzes the two sequential steps in the biosynthesis of PG from arachidonic acid, and plays a critical role in the inflammatory response. Our data showed that COX-2 protein expression was significantly induced by LPS stimulation, but treatments of LPS-stimulated murine macrophages with 1, 2 and 3 produced a remarkable downregulation of COX-2 expression. Nevertheless, no changes were observed after treatment with 7 and 8 **(Chapter IV. Figure 4)**.

LPS can trigger groups of different TLR especially TLR-4, starting downstream signaling cascades, including MAPK and the NF-κB pathways. NF-κB, as a dimeric transcription factor exists in the cytoplasm as an inactive complex with the inhibitory protein IkBα. When cells are challenged with proinflammatory stimuli, for example LPS, IkBα undergoes phosphorylation and subsequently ubiquitination, allowing NF-κB to translocate to the nucleus. As a result, NF-κB binds to kB enhancer elements present in the promoter region of many proinflammatory genes, for instance, iNOS and COX-2 (Lee and Surh, 2012). Our results show that pretreatment with 2 and 3 significantly prevented IkBα degradation after LPS stimulus. However, no significant changes were observed in IkBα expression after 1, 7, and 8 treatments **(Chapter IV. Figure 5).** Others researches have shown that anti-inflammatory activity by EVOO isolated phenols is mediated, at least in part, by NF-κB signaling. For instance, 1 at 25-100 μM has been demonstrated to block NF-κB intracellular oxidative stress attenuating iNOS and COX-2 expressions in human monocytes (Zhang et al., 2009). This capacity to regulate NF-κB genes was also described in J774 murine macrophages and in N2a cells against amyloid-β-induced toxicity (Maiuri et al., 2005; St-Laurent-Thibault et al., 2011). Nevertheless, our data are in agreement with another previous study in which no significant changes after co-incubation with 1 in HT-29 human cells were observed (Cárdeno et al., 2013). Moreover, a recent paper has showed that HTy suppressed NO production by decreasing iNOS gene expression through a mechanism independent of the NF-κB signaling pathway (Takeda et al., 2014). Maybe these discrepancies could be explained in terms of different phenol concentrations and changes on cell experimental conditions.

As above mentioned, LPS can induce MAPK activation. MAPK are a family of serine–threonine kinase enzymes which orchestrate the recruitment of gene transcription, protein biosynthesis and differentiation and allow cells to respond to oxidative stress and inflammatory stimuli from their extracellular environment (Munoz and Ammit, 2010). In fact,

MAPK have been shown to play important roles in iNOS and COX-2 upregulation induced by various stimuli in mammalian cells. Nevertheless, our findings demonstrated no significant changes in p38 and JNK protein phosphorylation after treatments, suggesting that MAPK signaling pathway does not make a consistent contribution in the anti-inflammatory effects of 2 and 3 in mouse peritoneal macrophages **(Chapter IV. Figure 6).**

In conclusion, this study establishes for the first time that the natural HTy derivatives 2 and 3 present in EVOO inhibit LPS-induced oxidative stress and inflammatory response via direct downregulation of NO generation. These protective effects seem to be due to a downregulation of iNOS and COX-2 protein expression via inhibition of NF-κB signaling pathway. Acylation of the side chain of 3,4-hydroxyphenylglycol 3 by catechol protection, acylation of aliphatic hydroxyl groups, and deprotection afforded diacylated 7 and 8, which show worse anti-inflammatory activity than the parent 3. Furthermore, 8 with the longer acyl chains, showed the worst behavior on the stimulated macrophages.

The implication of this study for nutrition and general health of the population recline in the possible use of natural HTy derivatives with better hydrophilic/ lipophilic balance, thus improving its pharmacodynamic and pharmacokinetic profiles, as new dietary supplements, however, further *in vivo* studies are necessary to fully understand the role of these EVOO phenols in the management of inflammatory-related diseases.

The objective of the **chapter V** was to determinate the potential protective role of HTy or HTy-Ac dietary supplementation in the prevention and development of SLE in a murine pristane-induced experimental lupus model. We have demonstrated for the first time that both HTy and HTy-Ac dietary supplementation modulated pristane induced SLE on mice, preventing renal inflammatory markers through NF-κB, STAT3 and MAPK signaling pathways.

SLE is a multisystem autoimmune disease in which immunological abnormalities, including increased production of proinflammatory cytokines and lymphocyte hyperactivity, are manifested in several organs, mostly in kidney and spleen (Kyttaris, 2010; Takeuchi et al., 2005). Th1 cytokines, such as TNF-α and IL-1β, are known to contribute directly to the inflammatory injury in SLE (Lourenço and Cava, 2009; Umare et al., 2014). In addition, TNF-α has a pivotal role in T cell apoptosis induction (Aringer and Smolen, 2003). Moreover, Th2 cytokines, like IL-6, have a remarkably value in lupus autoantibody production (Richards et al., 1999) whereas that IL-17 cytokines play an important role in immune response amplification via local production of chemokines and cytokines and monocytes and neutrophils recruitment, aggravating inflammation and injury in target organs (Li et al., 2015). In this study, we found lower levels of induced TNF-α, IL-1B, IL-6 and

IL-17 in supernatants from splenocytes and peritoneal macrophages from pristane mice fed with HTy and HTy-Ac supplemented diets in comparison with pristane mice fed with standard diet **(Chapter V. Figure 2)**, suggesting that dietary intake of HTy and HTy-Ac contribute to reduce the inflammatory response by suppressing the production of Th1, Th2 and Th17 cytokines in pristane-treated SLE mice.

PGE_2 plays a pivotal role in the development of inflammatory responses and tissue injuries, acting as a mediator of Th1/Th2 nature of immune response (Chae et al., 2008). Consequently, lower levels of PGE_2 were found in LPS-stimulated splenocytes and peritoneal macrophages from pristane mice fed with HTy and HTy-Ac diets compared to those from pristane mice fed with standard diet **(Chapter V. Figure 3A and 3B)**. Lupus nephritis appears in approximately 60% of lupus patients and it is one of the main causes of SLE morbidity and mortality. Accordingly with the results found in splenocytes and macrophages, HTy and HTy-Ac enriched diets reduced not only PGE_2 production in kidney but also mPGES-1 protein expression, a key enzyme in PGE_2 biosynthesis **(Chapter V. Figure 3C)**. Therefore, the regulation of these proinflammatory biomarkers might represent a potential target to modulate kidney damage by HTy and HTy-Ac dietary intake.

On the other hand, the generation of ROS has a wide impact in acute and chronic kidney injuries in patients with lupus nephritis. Nrf2, the major regulator of the antioxidant response, constitutes a primary cellular defence mechanism with a protective role by scavenging ROS and regulating HO-1 expression (Herrada et al., 2012; Jiang et al., 2014). It is known that HO-1 induction therapy ameliorates lupus nephritis, probably by multiple mechanisms including NO synthesis suppression, inhibition of antibody production and modulation of cytokine production (Takeda et al., 2004). In the present study, Nrf2 and HO-1 proteins expressions were decreased in pristane group but HTy and HTy-Ac dietary supplementation could restore Nrf2/HO-1 expression, conferring a potential beneficial effect of HTy and HTy-Ac supplementation in this SLE model **(Chapter V. Figure 4A)**. In addition, Nrf2 inhibits SLE development of lupus by suppressing NF-κB-mediated inflammatory response (Jiang et al., 2014). Therefore, we investigated whether dietary HTy and HTy-Ac might prevent the activation of NF-κB signaling pathway. In our study, kidney IκBα protein expression decreased after pristane injection. Conversely, kidneys from pristane mice fed with HTy or HTy-Ac showed similar IκBα expression levels than sham group. In accordance with these results, p65 translocation was also reduced in HTy or HTy-Ac groups compared to pristane group mice **(Chapter V. Figure 4B)**.

STAT3 inhibition represents a potential therapeutic target in SLE because its activation is critical for T:B cell cross-talk, Th17 generation and T cell migration, playing a

central role in lupus pathogenesis and particularly in lupus nephritis (Edwards et al., 2015). Importantly, MAPK phosphorylate STAT3, leading to STAT3 activation by phosphorylation on tyrosine residues resulting in the formation of STAT dimers that translocate into the nucleus to bind specific DNA sequences (Wang et al., 2005). Our findings show that STAT3 and MAPK phosphorylation are increased in kidneys from SLE pristane mice but dietary HTy or HTy-Ac supplementation significantly reduced their phosphorylation **(Chapter V. Figure 5A and 5B)**.

Nowadays, nutritional therapy, including diet modification and the use of nutritional supplements, could be a promising way to approach SLE due to the potential prophylactic effects without the side effects of the classic pharmacological therapy and its possible contribution to reduce comorbidities and improve quality of life in SLE patients (Greco et al., 2013). Diet quality in SLE patients is important since these patients present higher risk for another diseases, which are directly influenced by diet. In this regard, phenolic compounds included in our daily diet have shown a broad spectrum of beneficial effects and bioactive properties that highlight their possible supportive role in primary and secondary prevention and management of SLE.

In accordance with the results of the present study, dietary HTy and HTy-Ac supplementation significantly reduced proinflammatory cytokines on peritoneal macrophages and splenocytes with a remarkable blockage of JAK/STAT, MAPK and NF-κB pathways, showing for the first time that the dietary intake of these phenolic compounds improve different immune-inflammatory markers on a SLE mice model. However, the full biological significance of these results on SLE is a key issue and further investigations are warranted to provide insights into the influence of these phenolic compounds and their beneficial effects on human SLE. Nevertheless, our results provide preliminary evidences that these phenolic compounds could exert benefits again autoimmune and inflammatory disorders such as SLE and provide a new dietary strategy in the prevention and management of SLE.

The focus of the **chapter VI** was to study the effects of HTy and HTy-Ac in an *in vitro* model of human keratinocytes and clarify the mediators and molecular mechanism involved.

Apart from their well-established anti-oxidant properties, EVOO polyphenols are also found to have another beneficial properties of medical interest including anti-atherogenic, antitumor, anti-inflammatory and immune-modulatory activities that appear to be only in part related to their antioxidant power (Rigacci and Stefani, 2016). Particularly, HTy and HTy-Ac have shown interesting prophylactic and preventive effects in

some cell and animal models of inflammation, including arthritis, inflammatory bowel disease and hypoxia–reoxygenation in brain (Rosillo et al., 2015; Sánchez-Fidalgo et al., 2015a). However, such effects have never been previously proven in models of inflammation of the skin. Thus, the present work constitutes the first evidence that HTy and HTy-Ac may constitute a possible preventive and palliative alternative in the treatment of inflammatory skin diseases.

In our study, both polyphenols showed preventive effects in the induced inflammatory response in a model of primary human keratinocytes. Thymic stromal lymphopoietin (TSLP) represents a key cytokine of the skin immune system, deeply associated with and a driver of inflammatory skin diseases (Kumari et al., 2014; Ziegler, 2012). In this sense, pre-treatment with HTy-Ac or HTy effectively prevented the IL-1β and TLR3 ligation mediated induction **(Chapter VI. Figure 1)** and effectively prevented the transcriptional induction of TSLP **(Chapter VI. Figure 2A)**. In humans, TSLP is produced in 2 isoforms with different immunomodulatory roles: a short isoform, expressed in skin in homeostatic conditions (but which can be further modulated during inflammation), and a long isoform, which is exclusively expressed under inflammatory or otherwise pathogenic conditions (Fornasa et al., 2015). Our results show that HTy-Ac and HTy were able to modestly decrease the induction of the short TSLP transcript and indeed decreased long TSLP mRNA after both 2 and 4 hours of IL-1β or TLR3-l stimulation **(Chapter VI. Figure 2B and 3A).** A major pathway by which TSLP expression is induced in inflammatory surroundings is by activation of NF-κB, for which four potential binding sites have been uncovered in the human TSLP promoter (Cultrone et al., 2013). In the presence of HTy and HTy-Ac, both the degradation of IκBα and the nuclear translocation of p65 were indeed restrained, so that higher levels of IκBα and lower nuclear levels of p65 were detected at both time points **(Chapter VI. Figure 4A and 4B).**

To gain further insight into the influence of EVOO phenols on NF-κB regulated gen and cytokines in the context of the skin, their gene expression was measured by RT-qPCR. HTy and HTy-Ac decreased proinflammatory gene levels of different proinflammatory cytokines (TNF-α, IL-6 and IL-8) and genes (COX-2) involved in the inflammatory balance of the skin **(Chapter VI. Figure 5 and 6**). Overall, we could emphasize that HTy seems to exhibit stronger anti-inflammatory properties than HTy-Ac in this inflammatory model in skin.

HTy is perhaps one of the largest studied EVOO polyphenols and it is identified as a viable candidate for further development of new drugs for the control of inflammation and immune response. In this regard, pre-treatment with HTy in monolayer cultures of human chondrocytes not only effectively prevented the accumulation of ROS, DNA damage and

cell death induced by H_2O_2 exposure, but also increased mRNA level of proinflammatory marker genes (Facchini et al., 2014). Furthermore, in an atopic dermatitis model in mice, HTy showed anti-inflammatory and antioxidant effects, controlling erythema intensity and dermatitis index and decreasing T-helper cells (TH1/TH2) producing cytokines in serum and skin biopsies but only in the presence of hydrocortisone (Hussain et al., 2014; Hussain et al., 2013).

On the other hand, HTy-Ac, found in EVOO at a proportion similar to HTy (depending on the variety of olive), has been less well studied than HTy, but it has recently aroused great interest among the current research on natural products. HTy-Ac showed to modulate the inflammatory response triggered by LPS in murine peritoneal macrophages, downregulating COX-2 expression and preventing IκBα degradation (Aparicio-Soto et al., 2015). Furthermore, HTy-Ac exhibited promising effects in different immune-inflammatory animal models. For example, HTy-Ac reduced colonic damage, decreased COX-2 and iNOS protein expression, downregulated JNK phosphorylation and nuclear translocation of p65 in a model of dextran sulfate sodium (DSS) induced acute colitis in mice (Sánchez-Fidalgo et al., 2015a). Besides, HTy-Ac reduced inflammatory markers in a model of murine arthritis, and downregulated JAK-STAT, MAPK and NF-κB signaling pathways. Conversely, in this same model the treatment with HTy had no beneficial effects (Rosillo et al., 2015), suggesting that the two polyphenols do could act through different mechanism in different inflammatory diseases.

In summary, our data constitute the first evidence that these EVOO phenols efficiently improve the inflammatory process in a model of human keratinocytes by decreasing TSLP production and gene expression as well as other proinflammatory gene levels probably through interference with the NF-κB pathway. Therefore, these EVOO polyphenols might be considered as promising compounds with anti-inflammatory effects, which could contribute in a close future to the management of inflammatory skin diseases including SLE.

Altogether, this thesis conclude that EVOO exerts preventive/palliative effects in the development of experimental SLE, with a remarkable role of its minor components such as PE and isolated phenolic compounds in its beneficial effects. Hence, EVOO may constitute a key functional food and its phenolic fraction as well as its isolated phenolic compounds might provide a potential nutraceutical complement in the management of SLE, owing to both its potential prophylactic effects without the side effects of classical pharmacology and its potential contribution to reducing comorbidities and improving

quality of life in patients with SLE, thereby offering a new promising therapeutic strategy in the management of this immune-inflammatory disease.

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CONCLUSIONES

Los resultados obtenidos en la presente tesis doctoral nos permiten concluir:

1. Una dieta elaborada con AOVE con alto contenido en componentes fenólicos redujo significativamente las alteraciones histológicas observadas en riñón en un modelo murino de LES inducido por pristano, en comparación con aquellos animales alimentados con dieta estándar, con aceite de girasol como fuente lipídica. Igualmente, se observaron menores niveles de MMP-3 en suero y PGE_2 en riñón. Además, la dieta elaborada con AOVE mejoró la expresión de diversas proteínas implicadas en el daño renal características del LES, así como la producción de citoquinas inflamatorias en esplenocitos y macrófagos peritoneales. Estos resultados sugieren que el tratamiento dietético con AOVE puede ejercer un papel preventivo/paliativo en el manejo del SLE.

2. La FP del AOVE disminuyó la producción de NO y citoquinas inflamatorias junto con la expresión génica y proteica de marcadores inmunoinflamatorios en monocitos humanos. Asimismo, la FP moduló la expresión de marcadores anti y proinflamatorios en macrófagos humanos. Estos resultados ponen de manifiesto el especial protagonismo de la FP en los efectos inmunomoduladores del AOVE, que podrían ser de especial interés en el abordaje de enfermedades inmunoinflamatorias.

3. La FP del AOVE disminuyó la frecuencia de células CD69+ y la producción de IFN γ, TNF-α, IL-6, IL-1β e IL-10 en controles sanos y pacientes con LES. También se detectaron significativos incrementos en la producción de IL-6, TNF-α, e IL-10 en dichos pacientes y en los niveles de IL-1β, cuya producción fue significativamente menor tras la estimulación con PHA comparado con controles sanos. Asimismo, la FP incrementó la producción de IL-10 en controles sanos pero la disminuyó en pacientes con LES. Asimismo, la FP incrementó la expresión de la proteína inhibitoria IκB y disminuyó la fosforilación de la proteína ERK en pacientes con LES y pacientes sanos. Estos resultados resaltan el posible papel antiinflamatorio e inmunomodulador de la FP del AOVE en la modulación de la producción de citoquinas inflamatorias y la activación de células T, probablemente a través de la vía del NF-κB.

4. En un modelo de macrófagos peritoneales murinos estimulados con LP, los compuestos fenólicos del AOVE, HTy e HTY-Ac exhibieron marcadas actividades antioxidantes, previniendo la formación de especies reactivas de oxigeno, y antiinflamatoria, disminuyendo los niveles de nitritos y la expresión de las proteínas iNOS y COX-2. En particular, HTy-Ac podría constituir una interesante alternativa al HTy debido a su mejor perfil farmacocinético y farmacodinámico.

5. En un modelo murino de LES inducido por pristano, la suplementación dietética con HTy o HTy-Ac redujo significativamente la producción de citoquinas proinflamatorias en riñón, macrófagos peritoneales y esplenocitos. Igualmente, las dietas con HTy e HTy-Ac minimizaron el daño renal inducido por pristano, disminuyendo la expresión de diversas proteínas implicadas en el daño renal través de diversas vías involucradas en el LES, sugiriendo que ambos compuestos fenólicos pueden representar una nueva opción en el tratamiento nutricional del LES.

6. En un modelo de inflamación en queratinocitos humanos HTy y HTy-Ac disminuyeron significativamente la producción y expresión génica de TSLP junto con los niveles de otros genes proinflamatorios (TNF-α, IL-6, IL-8 y COX-2), probablemente a través de la capacidad de ambos compuestos para modular la actividad de la vía del NF-κB,, mediante la prevención de la degradación de la proteína inhibitoria IκBα y la translocación nuclear de la proteína p65. Nuestros resultados indican que ambos compuestos fenólicos mejoran el balance inflamatorio en este modelo de inflamación, y podrían constituir una nueva posibilidad terapéutica para el abordaje de enfermedades inflamatorias de la piel, como es el caso del LES.

Abstract

Systemic lupus erythematosus (SLE) can be defined as a chronic inflammatory and autoimmune disease that can affect multiple organ systems, including skin, joints, kidneys and the brain, among others (Noble et al., 2016). SLE is characterised by a deposition of immune complexes, formed in large amounts as antinuclear antibodies bind to the abundant nuclear material in blood and tissues, along with disturbances in both innate and adaptive immunity manifest by disorders in cytokines, apoptotic cell clearance, B-cell immunity, and T-cell signaling (Crispín et al., 2010; Lisnevskaia et al., 2014). It is also characterised by its clinical and pathogenic complexity, difficult diagnosis and the high number of complications that can affect the patients´ quality of life (Petri et al., 2012).

SLE affects multiple systems and its presentation and course are highly variable, ranging from indolent to fulminant. The most common clinical manifestations include fatigue, loss of appetite and weight, cutaneous lesions (mainly malar rash), arthritis, serositis (pleuritis and/orpericarditis), renal or central nervous system involvement and hematological manifestations (cytopenias) associated with several autoantibodies, particularly antinuclear antibodies (ANA)(Petri et al., 2012).

Nowadays, the overall aim of SLE therapy is to control disease activity. Mild activity can be managed with non-steroidal anti-inflammatory drugs or low-dose glucocorticoids, but more severe manifestations require more advanced treatment (Bertsias et al., 2012; Jordan and D'Cruz, 2015; Kuhn et al., 2015). Typical SLE management includes the use of antimalarial drugs (mainly hydroxychloroquine and chloroquine), immunosuppressive agents, biological agents and some adjunctive therapies following international recommendations (Kuhn et al., 2015). Nevertheless, SLE management remains complicated owing to the biological heterogeneity between patients and the lack of safe and specific targeted therapies. Thus, the search for new therapeutic targets and strategies that can act more selectively on certain routes or biological processes and improve the course of disease or reverse the outbreak phase without generating collateral damage to unaffected tissues and organs is the pillar underlying current research in SLE.

Nutritional therapy, including diet modification and the use of nutritional supplements, could be a promising way to approach SLE owing to both its potential prophylactic effects, without the side effects of the classic pharmacological therapy, and its possible contribution to reducing comorbidities and improving the quality of life of patients with SLE (Greco et al., 2013). In this regard, there is evidence that dietary factors can contribute to the geoepidemiology of autoimmune diseases (Selmi, 2010). Recent studies have suggested that the traditional Mediterranean diet might confer protection from certain chronic diseases related to oxidative stress, inflammation and the immune

system. The beneficial effects of the Mediterranean diet have been proven in cancer, cardiovascular diseases, obesity and arthritis (Casas et al., 2014; Cárdeno et al., 2013; Toledo et al., 2013). Likewise, epidemiological data have shown a decreased prevalence of rheumatic diseases in Mediterranean countries when compared with Northern Europe, and some clinical trials have demonstrated that the Mediterranean diet improves rheumatic symptoms and decreases the use of anti-inflammatory drugs and classic pharmacotherapy-related side effects (McKellar et al., 2007; Miggiano and Gagliardi, 2005).

In comparison with other healthy diets, olive oil is located at the centre of the Mediterranean diet pyramid as the principal source of dietary lipids, being described as a key bioactive food because of its high nutritional quality and its particular composition, especially relevant in the case of extra virgin olive oil (EVOO)(Bach-Faig et al., 2011; Puertollano et al., 2010). EVOO is the olive oil obtained from the fruit of the olive tree (*Olea Europea* L.) solely by mechanical or other physical preparation under conditions that do not alter its natural composition. Traditionally, the beneficial effects of EVOO have been ascribed to its high content of MUFA, particularly oleic acid, which has shown multiple beneficial properties (Bermudez et al., 2011; Waterman and Lockwood, 2007). However, in recent years converging evidence indicate that EVOO minority components contribute to the benefits of its consumption. In this sense, olive oil polyphenols such as hydroxytyrosol (HTy), tyrosol, hydroxytyrosyl acetate (HTy-Ac) and oleuropein may be responsible for some of the beneficial properties of EVOO activities that appear to be only in part related to the antioxidant power of these molecules (Rigacci and Stefani, 2016).

Despite the broad range of evidences of the potential benefits of EVOO in immune-inflammatory related diseases, the particular effects of EVOO and its potential bioactive components as well as the specific mechanisms by which them may exert their anti-inflammatory and immunomodulatory effects in SLE are still not elucidated.

Therefore, the **objectives** of this thesis were:

1. To determinate the potential beneficial effects of EVOO in a model of SLE induced by pristane (2,6,10,14-tetramethylpentadecane) in BALB/c mice and explore the molecular mechanism and signaling pathways involved.
2. To evaluate the role of the phenolic fraction (PE) of EVOO and elucidate molecular mechanism and signaling pathways on:
    2a. Monocytes and macrophages from healthy donors
    2b. Peripheral blood mononuclear cells (PBMC) isolated from patients with SLE and healthy donors.

3. To investigate the potential effects of HTy and HTy-Ac, phenolic compounds from EVOO:

   3a. To evaluate the anti-inflammatory effects of HTy, HTy-Ac and other phenolic compounds of EVOO in an *in vitro* model of murine peritoneal macrophages.

   3b. To investigate the effects of HTy and HTy-Ac enriched diets in a model of SLE induced by pristane (2,6,10,14-tetramethyilpentadecane) in BALB/c mice and explore the molecular mechanism and signaling pathways involved.

   3c. To determinate the effects of HTy and HTy-Ac in an *in vitro* model of human keratinocytes isolated from skin of healthy donors. Study of the gene modulation, cytokine production and signaling pathways involved.

Results and discussion

1. Dietary extra virgin olive oil attenuates kidney injury in pristane-induced SLE model via activation of HO-1/Nrf2 antioxidant pathway and suppression of JAK/STAT, NF-κB and MAPK activation [Aparicio-Soto *et al.* (2016) J Nutr Biochem 27, 278-88].

BALB/c Mice were randomized into four experimental groups: (1) sham sunflower diet group were fed with a diet elaborated with a marketable sunflower oil, (2) pristane sunflower diet group, fed with a diet elaborated with a marketable sunflower oil (3) sham EVOO diet group were fed with a diet made with a marketable EVOO picual variety and (4) pristane EVOO diet group, fed with a diet made with a marketable EVOO picual variety. At 16 weeks of age, SLE was induced by a pristane injection according to the procedure described by Satoh and Reeves (Satoh and Reeves, 1994). At the end of the experimental period (24 weeks), animals were euthanized and specimens including blood, spleen, thymus and kidney were collected.

Our results revealed that EVOO, as the lipid component of the diet, had a therapeutic role in the development of SLE in female BALB/c in comparison with those pristane mice fed with sunflower oil diet. This beneficial effect was well correlated to an improved renal damage score, as reflected by milder proteinuria and a minor inflammatory mononuclear cells infiltration in renal interstitium and reduced paw swelling. Moreover, dietary EVOO ameliorated proinflammatory cytokines production, reducing tumour necrosis factor (TNF)-α, interleukin (IL)-6, IL-10 and IL-17 production in lipopolysaccharide (LPS)-stimulated splenocytes in comparison with those animals fed with sunflower oil. Besides, metalloproteinase (MMP)-3 serum levels were increased in pristane-treated mice fed with sunflower diet whereas those pristane-treated animals which

received EVOO diet expressed similar serum MMP-3 levels to those described in control mice contributing to minimize renal damage produced by pristane induction.

In addition, we demonstrated that pristane injection increased kidney phosphorylation of p38, JNK and ERK mitogen-activated protein kinases (MAPK) and signal transducer and activator of transcription 3 (STAT3) in those animals fed with sunflower diet, and it was directly related to the severity of renal damage. On the contrary, EVOO diet significantly reduced both MAPK and STAT3 activation in kidney. Moreover, EVOO diet ameliorated PGE_2 production and microsomal prostaglandin E synthase (mPGES)-1 protein expression in kidney from pristane-treated mice. Our data also indicate that nuclear factor E2-related factor 2 (Nrf2) and heme oxygenase (HO-1) protein expressions were upregulated in those mice fed with EVOO and the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway was drastically ameliorated after EVOO diet.

In summary, our findings suggest that dietary EVOO has protective effects, alleviating pristane-induced SLE in mice by reducing levels of inflammatory mediators and activating HO-1/Nrf2 antioxidant pathway by blocking janus kinase (JAK)/STAT, NF-κB and MAPK-mediated inflammatory response and it may constitute a potential functional food with a preventive/palliative role in the management of SLE.

2a. Potential involvement of the phenolic fraction of extra virgin olive oil in systemic lupus erythematosus by regulating PPAR-γ dependent monocyte activation

We evaluated the effects of dietary EVOO on peritoneal macrophages from pristane-induced SLE mice as well as the immune-inflammatory effects of the PE of EVOO on human primary monocytes and monocyte-derived macrophages focusing on cytokines production and molecular mechanisms. Sixty mice were randomized into four experimental groups and fed with diets elaborated with marketable sunflower oil or marketable EVOO picual variety. SLE was induced by pristane injection according to the procedure previously described (Satoh and Reeves, 1994). After 6 months of SLE induction, mice were sacrificed and peritoneal macrophages were collected and cultured. Study subjects were recruited at "Virgen del Rocío" University Hospital (Seville, Spain) for blood collection and isolation of human monocytes.

Modulation of proinflammatory mediators in macrophages is considered one of the strategies to develop therapeutic compounds against several inflammatory diseases. (Chen et al., 2015). In our study, EVOO diet decreased nitric oxide (NO) production and TNF-α, IL-6 and IL-17 levels on LPS-stimulated peritoneal macrophages from pristane-

induced lupus mice in comparison with those animals fed with sunflower diet. Additionally, PE treatment significantly reduced NO with a significant decrease of inducible nitric oxide synthase (iNOS) protein and NOS-2 gene expression and decreased Toll-like receptor (TLR)-4 mRNA levels as well as the gene expression of TNF-α, IL-1β and IL-6 proinflammatory cytokines on LPS-activated human monocytes. Some studies have shown the possible therapeutic benefits of peroxisome proliferator-activated receptor (PPAR)-γ agonists in SLE (Venegas-Pont et al., 2009). According to our results, PE treatment was able to significantly increased PPAR-γ protein expression as well as PPAR-γ mRNA levels on human monocytes.

Additionally, PE treatment downregulated the gene expression of M1 macrophages markers and up regulated M2 molecules on M0 macrophages suggesting that the PE from EVOO prevents the classical macrophages activation by LPS and interferon (IFN)-γ regulating inflammatory cytokine production and polarized M0 macrophages into M2 macrophages contributing to EVOO anti-inflammatory activities. Besides, PE decreased "classical" monocytes but increased "non-classical" monocytes and produced a significant reduction of chemokine receptor type 2 (CCR2)+ cells and CCR2 gene expression levels on LPS-treated classical monocytes.

Our findings outline that EVOO and particularly its PE have a significant therapeutic potential for the treatment of immune-inflammatory diseases such as SLE, through the modulation of monocytes/macrophages over-activation, decreasing NO and cytokine production as well as several immune-inflammatory markers.

2b. The phenolic fraction of extra virgin olive oil modulates the activation and the inflammatory response of T cells from patients with systemic lupus erythematosus and healthy donors

In this study we investigated the *in vitro* effects of the PE from EVOO on PBMC from patients with SLE and healthy subjects, as well as the possible underlying mechanisms responsible for these potential effects. PBMC were isolated from healthy donors and patients with SLE, meeting the American College of Rheumatology criteria for SLE (Tan et al., 1982), recruited in the Connective Tissue Disease Unit of the University Hospital "Virgen del Rocío" (Seville, Spain). All patients were women without clinical activity (SLEDAI <4).

We found a remarkable increase of IL-6 production in patients with SLE compared with healthy subjects. Moreover, IL-6 levels were significantly increased in both groups after phytohaemagglutinin (PHA) stimulation, whereas PE treatment was able to prevent this induced cytokine production. In addition, the *in vitro* treatment with PE positively regulated the induced production of IFN-γ on stimulated PBMC from patients with SLE and healthy

donors, contributing to equilibrate this induced immune-inflammatory imbalance. We also found significant differences in TNF- α production between patients with SLE and healthy donors: the levels of this proinflammatory cytokine appeared significantly increased in unstimulated and PHA-stimulated cells from patients with SLE compared with healthy donors. Besides, PE treatment was able to decrease in a significant way this induced production in both groups. Our study also showed that PHA produces a significant increase of IL-10 levels that was significantly elevated in patients with SLE when compared with healthy donors. Moreover, PE treatment showed distinctive activities between groups: in healthy donors IL-10 levels were enhanced after PE treatment whereas in patients with SLE its levels were significantly decreased.

CD69 is thought to be among the earliest surface markers of T-cell activation, and its changes are correlated with SLE disease activity (Draborg et al., 2014). In our study, we described that the PE from EVOO significantly decreased CD69 induced expression levels on CD4+ T lymphocytes from SLE patients, with a similar but dose-dependent effect on CD4+ T lymphocytes from healthy donors.

Moreover, our study showed that the expression of IκBα was significantly increased by PE treatment after PHA stimulation in patients with SLE as well as healthy donors, with similar results in both groups, suggesting that the activity of PE might be related to its capacity to modulate NF-κB activation and it may be an important mechanistic linked to the ability of diet to offset immune deregulation.

Altogether, this study showed that the PE from EVOO has an anti-inflammatory and immune-modulatory role in human PBMC, contributing to modulate the cytokine imbalance in patients with SLE and it might therefore be considered as a potential dietary strategy in SLE management.

3a. Naturally occurring hydroxytyrosol derivatives: hydroxytyrosyl acetate and 3,4-dihydroxyphenylglycol modulate inflammatory response in murine peritoneal macrophages. Potential utility as new dietary supplements [Aparicio-Soto *et al*. (2015) J Agric Food Chem 28;63 (3), 836-46].

This work evaluated the effects of two EVOO phenols, HTy-Ac and 3,4-dihydroxyphenylglycol, as well as two new acyl derivatives of 3,4-dihydroxyphenylglycol, on LPS-stimulated murine peritoneal macrophages in comparison with HTy. HTy-Ac was described for the first time in olive oil by Brenes *et al.* (Brenes et al., 1999) and it is found in most Spanish virgin olive oil. Moreover, HTy-Ac is more soluble in the lipophilic phases than

HTy, due to the presence of an ester group. Therefore, this increased lipophilicity suggests a better intestinal absorption than free HTy.

Our findings showed that these phenolic compounds, mainly compounds HTy-Ac and 3,4-dihydroxyphenylglycol, exhibited significant antiradical and antioxidant activities and prevented the progression of cellular damage induced by LPS, acting as effective antioxidants. The stimulation of macrophages by LPS induces the transcription of iNOS gene and the generation of large amounts of NO, which acts as an intracellular messenger modulating the formation of endogenous reactive oxygen species and orchestrating the inflammatory response (Li et al., 2012). In our study, we found that HTy-Ac and 3,4-dihydroxyphenylglycol significantly reduced both NO production and iNOS expression induced by LPS.

Our data also showed that cyclooxygenase (COX)-2 protein expression was significantly induced by LPS stimulation, but pretreatments with HTy-Ac and 3,4-dihydroxyphenylglycol produced a remarkable downregulation of COX-2 expression. In addition, our results show that the pretreatment with HTy-Ac and 3,4-dihydroxyphenylglycol significantly prevented IkBα degradation after LPS stimulation. However, there were no significant changes in p38 and JNK phosphorylation after the pretreatment with these phenolic compounds, suggesting that this signaling pathway does not make a consistent contribution in the anti-inflammatory effects of HTy-Ac and 3,4-dihydroxyphenylglycol in mouse peritoneal macrophages.

In conclusion, this study establishes for the first time that the natural HTy derivatives HTy-Ac and 3,4-dihydroxyphenylglycol inhibit LPS-induced oxidative and inflammatory response via direct downregulation of NO generation. These protective effects seem to be due to a downregulation of iNOS and COX-2 protein expression via inhibition of NF-κB signaling pathway.

3b. Dietary hydroxytyrosol and hydroxytyrosyl acetate supplementation prevent pristane-induced systemic lupus erythematous in mice. [Aparicio-Soto *et al.* (2016). Accepted by J Funt Foods].

Three-month old mice were randomized into four experimental groups: (1) sham group fed with standard diet, (2) pristane group fed with standard diet, (3) pristane hydroxytyrosol group (HTy) fed with standard diet supplemented with hydroxytyrosol (100 mg/kg diet), and (4) hydroxytyrosyl acetate group (HTy-Ac) fed with standard diet supplemented with hydroxytyrosyl acetate (100 mg/kg diet). SLE model was induced and

performed by the procedure previously described by Satoh *et al*. (Satoh and Reeves, 1994).

In this work, we found lower levels of TNF-α, IL-1B, IL-6 and IL-17 in splenocytes and peritoneal macrophages from pristane mice fed with HTy and HTy-Ac supplemented diets in comparison with pristane mice fed with standard diet, suggesting that dietary intake of HTy and HTy-Ac contribute to reduce the inflammatory response by suppressing the production of Th1, Th2 and Th17 cytokines in pristane-treated SLE mice. Furthermore, lower levels of PGE_2 were found in LPS-stimulated splenocytes and peritoneal macrophages from pristane mice fed with HTy and HTy-Ac diets compared to those from pristane mice fed with standard diet. Accordingly with these results HTy and HTy-Ac enriched diets also reduced PGE_2 production and mPGES-1 protein expression in kidney.

Nrf2 and HO-1 proteins expressions were decreased in pristane group but HTy and HTy-Ac dietary supplementation could restore Nrf2/HO-1 expression, conferring a potential beneficial effect of HTy and HTy-Ac supplementation in this SLE model. Moreover, kidney IκBα protein expression decreased after pristane injection but kidneys from pristane mice fed with HTy or HTy-Ac showed similar IκBα expression levels than sham group. In accordance with these results, p65 translocation was also reduced in HTy and HTy-Ac groups compared to pristane group mice. Additionally, STAT3 and MAPK phosphorylation were increased in kidneys from SLE pristane mice but dietary HTy and HTy-Ac supplementation significantly reduced these induced phosphorylation.

In summary, dietary HTy and HTy-Ac supplementation significantly reduced proinflammatory cytokines on peritoneal macrophages and splenocytes from pristane SLE mice with a remarkable blockage of JAK/STAT, MAPK and NF-κB pathways, showing for the first time that the dietary intake of these phenolic compounds improve different immune-inflammatory markers on this SLE model.

3c. Olive oil-derived phenols exert effective anti-inflammatory properties by interference with the NF-κB pathway and TSLP production in human keratinocytes

The present study was designed to evaluate the potential effects of HTy and HTy-Ac in an *in vitro* model of human keratinocytes and clarify the mediators and molecular mechanism involved. Human keratinocytes were isolated from skin of healthy donors and propagated according to a published protocol with several modifications (Artuc et al., 2002). Then, HTy-Ac or HTy were added at concentrations ranging 12.5 to 100 μM and cells were stimulated for different time points.

The pretreatment with HTy-Ac or HTy effectively prevented the mediated production of the cytokine thymic stromal lymphopoietin (TSLP) after 18 h of stimulation. Indeed, while TSLP mRNA expression was remarkably induced following stimulation after 2 and 4 h of incubation, HTy-Ac or HTy effectively prevented the transcriptional induction of TSLP. We also studied the two isoforms of TSLP separately. Our results show that expression of the short isoform, which is expressed in skin in physiological conditions, is also increased by IL-1β or TLR3-l stimulation after 2 and 4 h, whereas HTy-Ac and HTy were able to modestly decrease the induction of the short TSLP transcript. On the other hand, the long isoform of TSLP is not expressed under steady-state conditions and is de novo elicited by inflammatory stimuli (Fornasa et al., 2015). In line with this, our study revealed that the long TSLP mRNA is indeed increased after both 2 and 4 h of IL-1β or TLR3-l stimulation but the pretreatment with HTy-Ac or HTy significantly countered this induction in both stimulation regimens.

A major pathway by which TSLP expression is induced in inflammatory surroundings is by activation of NF-κB. In our study after 15 or 30 m of stimulation with IL-1β, there was an expected degradation of IκBα, the inhibitor of NF-κB, and a nearly simultaneous translocation of the NF-κB member p65 to the nucleus. On the contrary in the presence of HTy and HTy-Ac, both the degradation and nuclear translocation were indeed restrained, so that higher levels of IκBα and lower nuclear levels of p65 were detected at both time points. Additionally, HTy or HTy-Ac were able to significantly inhibit the induction of TNF-α, IL-6 and IL-8 after the stimulation with IL-1β or TLR3-l. Moreover, HTy or HTy-Ac significantly inhibited COX-2 mRNA levels after the stimulation with IL-1β.

Taken together, our data provide evidence that these EVOO phenols efficiently improve the inflammatory process in a model of human keratinocytes by decreasing TSLP production and gene expression as well as other proinflammatory gene levels probably through interference with the NF-κB pathway. Therefore, these EVOO polyphenols might be considered as promising compounds with anti-inflammatory effects, which could contribute in a close future to the management of inflammatory skin diseases.

Conclusion

Altogether, our results suggest that EVOO exerts preventive effects in the development of experimental SLE, with a remarkable role of its minor components such as PE and isolated phenolic compounds in its beneficial effects. Hence, EVOO may constitute a key functional food and its phenolic fraction as well as its isolated phenolic compounds might provide a potential nutraceutical complement in the management of SLE, owing to both its potential prophylactic effects without the side effects of classical pharmacology

and its potential contribution to reducing comorbidities and improving quality of life in patients with SLE, thereby offering a new promising therapeutic strategy in the management of this immune-inflammatory disease.

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